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## PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA

Ayman E. Ismail

University of Nebraska-Lincoln, [ismailo80@yahoo.com](mailto:ismailo80@yahoo.com)

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PURIFICATION OF FIBRINOGEN FROM  
HUMAN PLASMA

By

Ayman Ismail

A THESIS

Presented to the Faculty of  
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# PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA

Ayman Ismail, M.S.

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Adviser: William H. Velandar

A solvent detergent treated fibrinogen was purified from human plasma by cryoprecipitation (cryo) followed by chemical precipitation using ethanol (EtOH) or ammonium sulfate (AS) as precipitating agents. Amounts of fibronectin (FN), factor XIII A-subunit (FXIIIA), factor XIII b-subunit (FXIIIB), and  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP) in the isolated fibrinogen were quantified. Thromboelastography (TEG) analysis was used to evaluate the clot strength of the isolated fibrinogen and to determine the ability of the ethanol and ammonium sulfate precipitations to eliminate the solvent detergent. Sodium dodecylsulfate-polyacrylamide gel analysis indicated that fibrinogen produced by each of these precipitation methods had similar purity. Quantitative western blot analysis revealed that fibrinogen produced by ammonium sulfate precipitation contained increased amounts of FN, FXIIIB, and  $\alpha_2$ -AP. TEG analysis showed that ammonium sulfate precipitated fibrinogen yielded a fibrin clot with the highest maximal strength. In addition, a single ethanol precipitation was sufficient to remove the solvent detergent while a single ammonium sulfate precipitation was not effective in removing the solvent detergent mixture Tri (n-butyl) phosphate (TNBP) and Triton X-100 as judged by the ability of the material to form a clot.

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## INTRODUCTION

### Fibrinogen

Fibrinogen (FI) is a central molecule in the blood coagulation cascade that is transformed by thrombin into fibrin which polymerizes forming a clot to prevent the loss of blood at the site of vascular injury. Furthermore, because it has multiple binding sites that can react with other proteins, fibrinogen plays important roles in many physiological and pathological processes including blood clotting, fibrinolysis, cell adhesion, inflammation, angiogenesis, atherogenesis, tumorigenesis, and wound healing [1]. Fibrinogen is a 340 kDa glycoprotein consisting of two identical subunits, each of which is made of three polypeptide chains termed  $A\alpha$ ,  $B\beta$ , and  $\gamma$  [2]. The three chains of fibrinogen differ in terms of their structures and functions [Table 1].

A series of disulfide bonds link fibrinogen polypeptide chains forming elongated 45 nm tri-nodular structures with distinct domains. These domains are arranged into three main structural regions: a central E, two distal D and the  $\alpha C$  regions [3-5]. The central E nodule is formed by the N-terminal portions of the six polypeptide chains. The distal D nodules, formed by the C-terminal portions of the  $B\beta$  and  $\gamma$  as well as a fraction of the  $A\alpha$  chains, are separated from the E region by coiled-coil regions [6, 7]. The C-terminal region of the  $A\alpha$ -chain extends out from the D domains and is called the  $\alpha C$  domains [8].

**Table 1: Properties of fibrinogen chains**

	<b><math>\alpha</math>-chain</b>	<b><math>\beta</math>-chain</b>	<b><math>\gamma</math>-chain</b>
<b>Molecular Weight</b> [9]	67 KDa	54 KDa	47 KDa
<b>Amino Acids</b> [2]	610	461	411
<b>Fibrinopeptide</b>	FpA	FpB	None
<b>Carbohydrate</b> [10,11]	None	2 oligosaccharide	2 oligosaccharide

Based on the composition of the  $\gamma$ -chain, human fibrinogen can be isolated into two major fractions, fibrinogen-1 and fibrinogen-2 [12, 13]. Fibrinogen-1 possesses two homo-dimeric  $\gamma$ -chains each composed of 411 amino acids while fibrinogen-2 contains two hetero-dimeric molecules one  $\gamma$ -chain and one  $\gamma'$ -chain [12, 14]. The  $\gamma'$ -chain accounts for 15% of plasma fibrinogen and is formed by the alternative processing at the exon 9-exon 10 boundaries of the primary mRNA transcript [15]. The inclusion of intron 9 in the messenger results in the replacement of 4 amino acids (AGDV) of the  $\gamma$ -chain with a 20 amino acid sequence that contains two sulfated tyrosines and several Asp and Glu residues. Therefore, the  $\gamma'$ -chain is longer and contains more anion groups than the  $\gamma$ -chain [16, 14].

Fibrinogen has the ability to react with numerous substances with important physiological consequences. For example, The C-terminal of the A $\alpha$ -chain ( $\alpha$ C domains) of fibrinogen has binding sites for  $\alpha_2$ -AP, plasminogen, tissue-type plasminogen activator (tPA), and PAI-2. Table 2 lists binding sites and physiological roles of some fibrinogen-protein interactions.

**Table 2: Fibrinogen binding Ligands**

<b>Ligands</b>	<b>Binding Site</b>	<b>Function</b>
<b>Fibronectin</b> [17]	A $\alpha$ -chain	Cell adhesion
<b>FGF-2, bFGF</b> [18]	-	Proliferation of endothelial cells
<b>Lipoprotein (a)</b> [19, 20]	A $\alpha$ -chain	Inhibit fibrinolysis
<b>Thrombin</b> [21]	$\gamma'$ -chain	Fibrinopeptide release
<b>Plasminogen</b> [22]	A $\alpha$ -chain	Enhance fibrinolysis
<b>FXIII</b> [23-25]	$\gamma'$ -chain	Stabilizing clot
<b><math>\alpha_2</math>-AP</b> [26]	A $\alpha$ -chain	Resist fibrinolysis
<b>Integrin</b> [2]	A $\alpha$ -chain	Cellular interactions
<b>Calcium</b> [27]	B $\beta$ and $\gamma$ -chains	Promote polymerization

Human fibrinogen heterogeneity results from modification at several different sites both during and after biosynthesis. The heterogeneity arises from alternative splicing [28], alteration of different amino acids by sulfation [29], phosphorylation [30], various degrees of glycosylation [31], proteolysis [32], and some genetic polymorphisms [33]. These mechanisms form over a million species of fibrinogen [34]. These heterogeneities can change fibrin matrix properties thus helping to explain the difference that exist between individuals and wound healing.

During coagulation, thrombin converts soluble fibrinogen into fibrin monomers, which then polymerize to form a network of fibrin fibers. This network is stabilized by thrombin activated FXIII to form an insoluble fibrin clot. Formation of fibrin begins

when thrombin binds to a substrate site in fibrinogen and cleaves fibrinopeptides A (FpA) and B (FpB) from the amino termini of the A $\alpha$  and B $\beta$  chains, respectively. The removal of FpA exposes the polymerization site “A” that contains the N-terminal sequence (Gly-Pro-Arg-Val) of the A $\alpha$ -chain, while the removal of FpB exposes the polymerization site “B” that contains the N-terminal sequence of the B $\beta$ -chain [35]. The polymerization site “A” interacts with the complementary binding site of the  $\gamma$ -chain, whereas the polymerization site “B” interacts with the complementary binding site of the  $\beta$  chain [36]. These interactions lead to the formation of half-staggered, double-stranded protofibrils that undergo lateral aggregation to form fibers [37]. Thrombin simultaneously converts factor XIII (FXIII) to the active form (FXIIIa) which catalyzes formation of covalent  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds between adjacent fibrin molecules [37-40]. The cross-linking occurs rapidly between Lys-406 of one  $\gamma$ -chain and Gln-398 of another  $\gamma$ -chain to form  $\gamma$ -dimers [41, 42, 25]. In addition, hetero-dimers cross-linked  $\alpha$ - $\gamma$  have been found in plasma fibrinogen [43].

Different techniques have been developed for the purification of fibrinogen from plasma materials. Cryoprecipitation is the most common method for isolating fibrinogen, which reduces the solubility of fibrinogen at lower temperature to prevent its denaturation. Compared to fresh frozen plasma, cryoprecipitate contains an increased percentage of fibrinogen. Cryoprecipitation involves freezing the citrated plasma at lower temperature, usually -20 °C or less for at least 12 hours. The frozen plasma is slowly thawed at 4 °C followed by centrifugation to isolate the fibrinogen precipitate. The concentration of fibrinogen produced by cryoprecipitation is between 8 to 30 mg/ml and

can be increased to 40 to 60 mg/ml using repeated freeze/thaw cycles [44]. An ultrafiltration procedure has been used to purify fibrinogen where platelet rich plasma is separated using an ultrafiltration chamber with a molecular weight cutoff of 30 kDa. Fibrinogen obtained using this method has a final concentration of 6 mg/ml and lower clottability due to large amount of fibrinogen being denatured [45]. Fibrinogen has also been isolated from human plasma by using chemical precipitation methods in which chemical agents such as ethanol, glycine,  $\beta$ -alanine, ether, or ammonium sulfate are used to precipitate fibrinogen from plasma [46-49].

The clinical use of fibrinogen from pooled human plasma has been previously associated with a high risk of transmission of plasma-borne infectious species such as HIV, HBV, and HCV. The improvements in viral testing and screening have reduced, but not eliminated this risk. Therefore, different techniques have been developed in order to inactivate pathogenic viruses in fibrinogen derived from human plasma. Dry heat treatment at 60-68 °C of plasma cryoprecipitate inactivates HIV but it does not prevent the transmission of HCV [50]. Plasma pathogens have been inactivated by treating fresh plasma with methylene blue followed by exposure to visible light. This approach is less effective versus non-enveloped viruses and results in approximately 20% fibrinogen loss [51]. Solvent detergent (SD) treatment is the most effective procedure for inactivating blood-borne lipid-enveloped viruses. Solvent detergent treatment inactivates viruses by dissolving their lipid envelope but it does not inactivate non-lipid enveloped viruses such as parvovirus or hepatitis A virus [50]. The treatment of cryoprecipitate with a combination of organic solvent, tri (n-butyl) phosphate (TNBP), and detergent (sodium cholate, Tween 80, or Triton X-100) has been shown to inactivate very large quantities of

HBV, HCV, and HIV while preserving the activity of the purified protein. Radosevich et al [52] showed that treating cryoprecipitate with a mixture of 0.3% TNBP and 1% tween 80 at 25 °C for 6 hours inactivated  $\geq 5.5 \log_{10}$  of HIV,  $\geq 5 \log_{10}$  of VSV virus, and  $\geq 6.5 \log_{10}$  of sindbis virus. Horowitz and coworkers [53] revealed that subjecting pooled plasma to a mixture of 1% TNPB and 1% Triton X-100 for 4 hours at 30 °C inactivates  $\geq 10^6$  CID<sub>50</sub> of HBV,  $10^5$  CID<sub>50</sub> of HCV, and  $10^{6.2}$  TCID<sub>50</sub> of HIV.

### **Factor XIII**

Factor XIII, activated in the latter stages of the blood coagulation cascade, is a pro-transglutaminase that exists naturally in an intracellular or plasma form [54, 55]. The plasma form is a tetramer composed of two A-subunits (FXIIIA) and two B-subunits (FXIIIB) with a molecular mass of ~83 and ~80 kDa respectively [56]. Structural investigation of the tetramer indicates that the two A-subunits are globular proteins surrounded by two long flexible B-subunits [57, 58]. There are non-covalent interactions between the two A-chains and appears to be a non-covalent bond between the A and B [59]. The A-subunit contains the active site of the enzyme while the B-subunit serves to transport and protect the catalytic A-subunit until it is required in catalysis [60, 61]. Under normal conditions, the human plasma concentration of FXIII is ~14 to 28 mg/L [62]. Whereas carbohydrate accounts for approximately 8.5% of the total molecular weight of FXIIIB, the FXIIIA contains no carbohydrate [Table 3].

**Table 3: Characteristics of FXIII subunits**

	<b>A-subunit</b>	<b>B-subunit</b>
<b>Molecular weight</b> [56]	83 kDa	80 kDa
<b>Residues</b> [63, 64]	731	641
<b>Carbohydrate</b> [65]	None	~8.5% of the total weight
<b>N-terminal</b> [59]	Acetylated serine	Glutamic acid
<b>Disulfide bonds</b> [63]	None	20
<b>Structure</b> [64]	Globular	Kinked strand
<b>Function</b> [66]	Catalyzes an acyl transfer reaction	Acts as a carrier for A-subunit
<b>Secreted by</b> [66]	Liver, monocytes and megakaryocytes	Liver
<b>Active site</b> [66]	Cys314	None

The primary structure of FXIII subunits has been determined through the aid of amino acid sequence analysis and cDNA cloning. The A-subunit is made of five distinct folded domains: an activation peptide of 37 amino residues that is cleaved off during thrombin activation of the enzyme, a  $\beta$ -sandwich containing residues 38-183, a catalytic core composed of residues 184-515, and two barrels comprised of residues 516-627 and 628-731, respectively [66]. While the catalytic core consists of both  $\alpha$ -helices and  $\beta$ -sheets, the  $\beta$ -sandwich and barrel domains contain only  $\beta$ -sheets [Fig 1]. There are several hydrogen bonds and salt bridges between the  $\beta$ -sandwich, the activation peptide, and the catalytic core of the first subunit with the  $\beta$ -barrel and catalytic core of the second subunit [67]. These non-covalent interactions help stabilize the overall structure. Although FXIIIA possesses nine cysteine residues, no disulfide bridges have been detected in the subunit. There are six potential N-glycosylation sites in FXIIIA but no carbohydrate residue has been found in the secondary structure [65].

FXIIIB is a modular protein built from 10 repetitive sushi domains. Each sushi domain contains approximately 60 amino acids held together by two disulfide bridges forming a  $\beta$ -sandwich tertiary structure [63]. Employing electron microscopy and sedimentation techniques, the B-subunit appears as a thin, flexible strand with random kinks whereas unbound FXIIIB appears as a monomeric structure [64].

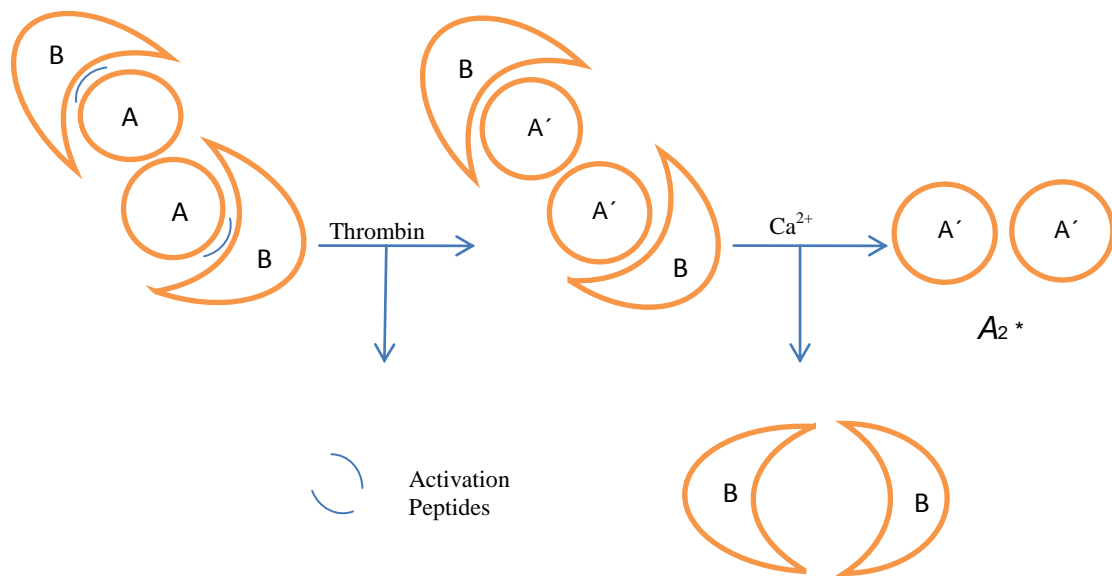
While plasma FXIII (pdFXIII) circulates as a tetramer, intracellular FXIII is a dimer composed of two A-subunits (FXIIIA<sub>2</sub>) each containing 730 amino acid residues [68]. This dimeric molecule has a molecular weight of approximately 160 kDa and mainly found in platelets, monocytes, and megakaryocytes [68, 63]. The primary structure of cellular FXIII is identical to plasma FXIIIA and the two molecules share the same immunochemical and electrophoretic properties.

FXIIIA catalyzes an acyl transfer reaction where glutamine residues serve as an acyl donor. This reaction proceeds through acylation and de-acylation intermediate steps to form an amide bond between  $\tau$ -carbonyl group of glutamine and  $\varepsilon$ -amino group of lysine [69]. Compared to other transglutaminase family members, FXIIIA has more restricted substrate specificity.

In the final stage of the blood coagulation cascade, the serine protease thrombin activates either form of FXIII in the presence of  $\text{Ca}^{2+}$ . The activation sequence begins when thrombin hydrolyzes the peptide bond at Arg37-Gly38 releasing the activation peptide from the N-terminal region of the FXIII [69, 70] [Fig 1]. Studies using radio crystallography showed that the released activation peptide did not bind thrombin but



remained attached to the FXIII molecule preventing the access of the substrate to the active site [58]. The activation sequence proceeds when  $\text{Ca}^{2+}$  binds to either the free activation peptide or the zymogen. This binding induces a conformational change that leads to the dissociation of the activated A-subunits from the B-subunits. As a result, the active site Cys-314 that was originally shielded by the activation peptide becomes unmasked and available for reaction [60, 71].



**Figure 1: Schematic diagram illustrates FXIII activation in plasma.** Removal of the activation peptide by thrombin weakens the binding of B-subunit to the truncated FXIIIA ( $A_2'B_2$ ). Conformational change induced by  $\text{Ca}^{2+}$  causes dissociation of  $A_2'B_2$  to generate FXIIIa [72].

Except for the dissociation of the B-subunit step, intracellular FXIII activation by thrombin follows the same mechanism as extracellular FXIII. Activation of intracellular FXIII is faster since the B-subunit of plasma FXIII suppresses activation of cellular FXIII

by thrombin thereby down-regulating pdFXIII cross-linking of fibrinogen [73]. The presence of fibrin speeds up the activation of pdFXIII by approximately one hundredfold [71, 74]. This is because the adsorption of thrombin and pdFXIII onto the fibrin (ogen) surface orient these molecules in a way that favors the hydrolysis of the peptide bond at Arg37-Gly38 by thrombin. Therefore, fibrin facilitates the activation of extracellular FXIII while the absence of B-subunit from intracellular FXIII inhibits fibrin catalysis [74].

The B-subunit of FXIII binds to the  $\gamma'$ -chain of fibrinogen thus heterodimeric fibrinogen (fibrinogen 2,  $\gamma/\gamma'$ ) serves as a carrier for FXIII in blood and also regulates its activity [75]. The presence of FXIII with fibrinogen in the plasma ensures that the formation of fibrin is associated with FXIII mediated cross-linking to generate a strong clot. FXIII cross-links homo-dimeric fibrinogen 1 molecules ( $\gamma/\gamma$ ) 3.5 times faster than hetero-dimeric fibrinogen 2 molecules ( $\gamma/\gamma'$ ) indicating that fibrinogen 2 bound FXIII is essential for inhibiting FXIII mediated cross-linking activity in plasma [74]. Fibrinogen also reduces the amounts of calcium required to activate the truncated FXIIIA dimer (FXIIIA<sub>2</sub>'). Besides thrombin, there are several other serine proteases such as endogenous platelet acid and calpain that can also activate FXIII by cleaving the Arg37-Gly38 peptide bond [76, 77].

Besides fibrin and  $\alpha_2$ -antiplasmin, FXIIIA has many other potential substrate such as plasminogen, lipoprotein (a), plasminogen activator inhibitor type 2 (PAI-2), thrombin-activatable fibrinolysis inhibitor (TAFI), fibronectin, and many other proteins [Table 4].

**Table 4: FXIII substrates**

<b>Substrate</b>	<b>Cross-linking Site</b>	<b>Cross-linking Targets</b>	<b>Function</b>
<b><math>\alpha_2</math>-antiplasmin</b> [78-80]	Gln2	Lys303 of $\alpha$ -chain	Inhibit fibrinolysis
<b>Fibrin (ogen)</b> <b><math>\alpha</math>-chain</b> [81-83]	Gln221 and 237 Lys208-Lys606	Itself and $\gamma$ -chains	Clot stabilization
<b>Fibrin (ogen)</b> <b><math>\gamma</math>-chain</b> [23-25]	Gln398, Gln399 and Lys406	Itself and $\alpha$ -chains	Clot stabilization
<b>Fibronectin</b> [84, 85]	Gln3	Itself, fibrin, and collagen	Wound healing; migration of cells into the clot
<b>Collagen</b> [84, 86]	Gln3	Fibronectin, and fibrin	Stabilization of extracellular matrix
<b>Von Willebrand Factor</b> [87, 88]	Gln3	Fibrin, collagen	Platelet adhesion to the clot
<b>Vitronectin</b> [89, 90]	Gln93	Fibrin, collagen	Platelet adhesion to the clot
<b>Factor V</b> [91, 92]	-	-	Increased thrombin generation at the clot surface
<b>Thrombospondin</b> [93]	-	Fibrin	Platelet adhesion to the clot
<b>Actin</b> [94, 95]	-	Fibrin	Clot reaction, stabilization of the platelet cytoskeleton
<b>TAFI</b> [96]	Gln2, Gln5, Gln292	Itself, fibrin	Resistance to fibrinolysis
<b>PAI-2</b> [97, 98]	-	Lys148, Lys230, Lys413 of fibrin $\alpha$ -chain	Resistance to fibrinolysis

FXIII has important physiological roles in hemostasis, angiogenesis, wound healing, and pregnancy [99, 100]. Plasma FXIII, also known as fibrin stabilizing factor, is essential for preserving hemostasis by performing the following functions: Increasing clot strength by cross-linking fibrin; inhibiting fibrinolysis by cross-linking  $\alpha_2$ -antiplasmin to the  $\alpha$ -chain of fibrin; inhibiting fibrinolysis by interfering with the adsorption of plasminogen into fibrin surface. FXIII is critical for creating a rigid, strong, and elastic clot needed to stop bleeding; therefore, FXIII deficiency results in severe bleeding [101-104]. FXIII also plays a central role in the events that lead to wound healing such as fibroblast proliferation, attachment of cells to substrate, and in collagen formation [105]. Studies showed that FXIII-deficient individuals have lower fibroblast growth rate than patients with normal FXIII [105, 106]. FXIII activates a series of biochemical reactions that assist in cell adhesion and without FXIII cells lose their regular shape due to cell contact inhibition. In addition, collagen is not produced in the absence of FXIII which delays wound healing [107].

Recombinant FXIIIA (rFXIII), which has similar structure and function as FXIII-A<sub>2</sub>, has been expressed in different cell types that include *Escherichia coli* [108], *Sacharomyces cerevisiae* [109, 110], and *Schizosacharomyces pombe* [111]. The numerous rFXIII crystal structures provided a wealth of information on catalytic activity as well as enzyme activation. Moreover, this structural information has been used to explain the consequences of some FXIII mutations [112].

## Alpha2-antiplasmin

Human  $\alpha_2$ -antiplasmin is the primary physiological inhibitor of the blood fibrinolytic plasmin.  $\alpha_2$ -AP is secreted by the liver as a single-chain glycoprotein of 464 amino acid residues and molecular weight of ~70 kDa with methionine as the N-terminus (Met-  $\alpha_2$ AP) [113, 114]. The plasma concentration of  $\alpha_2$ -AP is ~70  $\mu$ g/ml with a half-life of 2.6 days [115-117]. During circulation, Met- $\alpha_2$ AP undergoes proteolytic cleavage between Pro12-Asn13 by circulating anti-plasmin cleaving enzyme (APCE), a proline-specific serine protease [118]. This results in the formation of a 452 amino acid derivative with N-terminal asparagine (Asn- $\alpha_2$ AP). Asn- $\alpha_2$ AP has a molecular weight of ~64 kDa and accounts for 70% of the  $\alpha_2$ -AP in plasma while Met-  $\alpha_2$ AP accounts for the remaining 30% [113, 114]. Some properties of Met-  $\alpha_2$ AP and Asn-  $\alpha_2$ AP are listed in table 5. The human  $\alpha_2$ -AP gene is located on chromosome 17, specifically 17pter-p12 and 17p13, and contains 10 exons and 9 introns [119]. The complete primary structure of human  $\alpha_2$ -AP has been deduced from the sequence of its cDNA.

Human  $\alpha_2$ -antiplasmin belongs to the serine protease inhibitor (serpin) family but unlike other serpins,  $\alpha_2$ -AP has an extensive C-terminal sequence of approximately 50 amino acids that contains a number of lysine residues that are important for plasmin inhibition and plasmin (ogen) binding [120]. In addition, the C-terminal region of  $\alpha_2$ -AP contains an RGD tri-peptide sequence (Arg426-Gly-Asp) that is essential for cell recognition and adhesion [121]. Using the RGD sequence, the C-terminal region of  $\alpha_2$ -AP binds to endothelial cell integrins. With the aid of X-ray structure, the C-terminal of

$\alpha_2$ -AP is shown to be positioned in the proximity of the reactive loop and binds strongly to the K1 and K4 domains of plasmin increasing the inhibitory effect of  $\alpha_2$ -AP [122-124].

**Table 5: Properties of  $\alpha_2$ -antiplasmin**

	Met- $\alpha_2$ AP	Asn- $\alpha_2$ AP
Molecular weight [113, 114]	67 KDa	64 KDa
Amino acids [113, 114]	464	452
N-terminal	Methionine	Asparagine
Percentage [113, 114]	30%	70%
Binding to fibrinogen [118]	Slower	3-13 times faster

Human  $\alpha_2$ -AP regulates the fibrinolysis process in three ways [125]:

- I. Inactivates plasmin by forming a complex with it [126].
- II. Prevents plasminogen adsorption to fibrin surface [127, 128].
- III. Increases fibrin resistant to fibrinolysis by cross-linking fibrin with the aid of FXIII [129-131].

In plasma,  $\alpha_2$ -AP inactivates plasmin rapidly with a second order reaction rate of  $2 \times 10^{-7} \text{ mol}^{-1} \text{ s}^{-1}$  [132]. The reaction of Lys448 and Lys464 residues in the carboxyl-terminal of  $\alpha_2$ -AP with 1-3 kringles of plasmin (ogen) changes the conformation of the Ser741 of plasmin which then binds to Arg376 of  $\alpha_2$ -AP to form the inactive irreversible plasmin- $\alpha_2$ AP complex [133, 134]. Moreover, Lys groups in both  $\alpha_2$ -AP and fibrin bind to the same active sites in plasminogen thereby  $\alpha_2$ -AP competitively inhibits the binding of plasminogen to fibrin. Free plasmin generated by tissue-type plasminogen activator in plasma has a short half-life ( $\sim 0.1$  sec) due to the rapid inactivation by  $\alpha_2$ -AP, while plasmin produced in fibrin surface has a relatively longer half-life ( $\sim 10$  to  $100$  sec)

because it is only slightly inactivated by  $\alpha_2$ -AP [135]. This indicates the importance of prior incorporation of  $\alpha_2$ -AP into fibrinogen for effective inhibition of fibrinolysis.

The cross-linking of  $\alpha_2$ -AP to fibrinogen by FXIII is the most effective way to inhibit endogenous fibrinolysis concurrent with fibrin formation and caused by activation of plasminogen-bound to fibrin [130, 136]. The cross-linking reaction occurs between either Gln14 of Met- $\alpha_2$ AP or Gln2 of Asn- $\alpha_2$ AP and Lys303 of  $\alpha$ -chain of fibrin. FXIII catalyzes transfer of acyl groups donated by  $\alpha_2$ -AP to the acceptor amine groups of fibrinogen. The cross-linking reaction of  $\alpha_2$ AP with fibrinogen progress rapidly to reaches a maximum plateaus with only 20-30% of the available Lys303 active sites being occupied [137-139]. This limited reactivity suggests that the reaction equilibrium favors dissociation of the cross-linked product or Lys303 active sites are unavailable for cross-linking reaction [140]. Asn- $\alpha_2$ AP cross-links fibrinogen 3-13 times faster than Met- $\alpha_2$ AP. Consequently, the ratio of the two forms of  $\alpha_2$ -AP plays a critical role in the regulation of fibrinolytic process [118].

While the molecular mechanism of plasmin inhibition in solution is well understood, the inhibition of plasmin on the fibrin surface still requires further studies. In addition to plasmin inactivation,  $\alpha_2$ -AP also inhibits tissue-type plasminogen activator (TPA) and urokinase. The physiological importance of  $\alpha_2$ -AP is best characterized by issues encountered by individuals deficient in this inhibitor. These patients experience uncontrolled fibrinolysis and recurrent severe hemorrhagic disorder [141, 142].

Previous studies have shown that the content of  $\alpha_2$ -AP in plasma-derived fibrinogen is substantial (1.2-1.8 moles  $\alpha_2$ -AP per mole pdFI), and that native FXIII catalyze its cross-linking in circulation [143].

## **Fibronectin**

Fibronectin is the second most abundant protein within the extracellular matrix (ECM) mediating its interactions with variety of cells. Fibronectin is multifunctional glycoprotein exists in a soluble form in plasma, and in an insoluble form in the ECM. Plasma fibronectin (pdFN) is synthesized by hepatocytes and secreted into plasma where it circulates at a concentration of approximately 300-400  $\mu\text{g/ml}$  [144]. Cellular fibronectin (cFN) is composed of covalently cross-linked multimers and synthesized by different cell types including fibroblasts, endothelial cells, chondrocytes, synovial cells, and myocytes [145].

Fibronectin is composed of two very similar polypeptides with a molecular weight of 220-250 kDa [146-148]. The polypeptides are linked by inter-chain disulfide bonds located close to the C-terminal region forming a dimer molecule [149, 150]. Using rotary shadowing electron microscopy the polypeptides appear as flexible extended V-shaped molecules or compact globular molecules [151]. Each polypeptide is made of multiple homologous modules called type I, II, and III (F1, F2, and F3) in series [152-154]. There are 12 type I modules, two type II modules, and 15-17 type III modules, which together accounts for ~90% of the fibronectin sequence. Type I modules contain ~45 amino acids held together by two disulfide bonds and located in the amino and



carboxyl termini regions of each subunit. Type II modules are composed of ~60 amino acids linked by two disulfide bonds and localized in the gelatin binding domain of the subunit. Type III modules consists of ~90 amino acids that are clustered together in the middle of the subunit and does not contain disulfide bonds [155]. The N-terminal region of fibronectin consist of five type I modules whereas the C-terminal contains three type I modules.

Fibronectin contains binding sites for different molecules that are localized along the length of the monomer enabling fibronectin to play an important role in many physiological processes [156]. Because it's rich in Arg-Gly-Asp tri-peptide sequences, fibronectin plays important roles in cell migration, growth, and differentiation [157]. Fibronectin incorporated into fibrin by FXIII mediates interaction of fibrin with cells or platelets during clot formation [158-160]. Bound fibronectin forms a three-dimensional matrix at the wound site that attracts different cell types and extracellular matrix proteins [161]. F1 (1-5) contains binding sites for heparin, fibrin, and cell surface proteins for pathogenic bacteria such as streptococcus dysgalactiae that play a putative mechanism for host by microorganism [162].

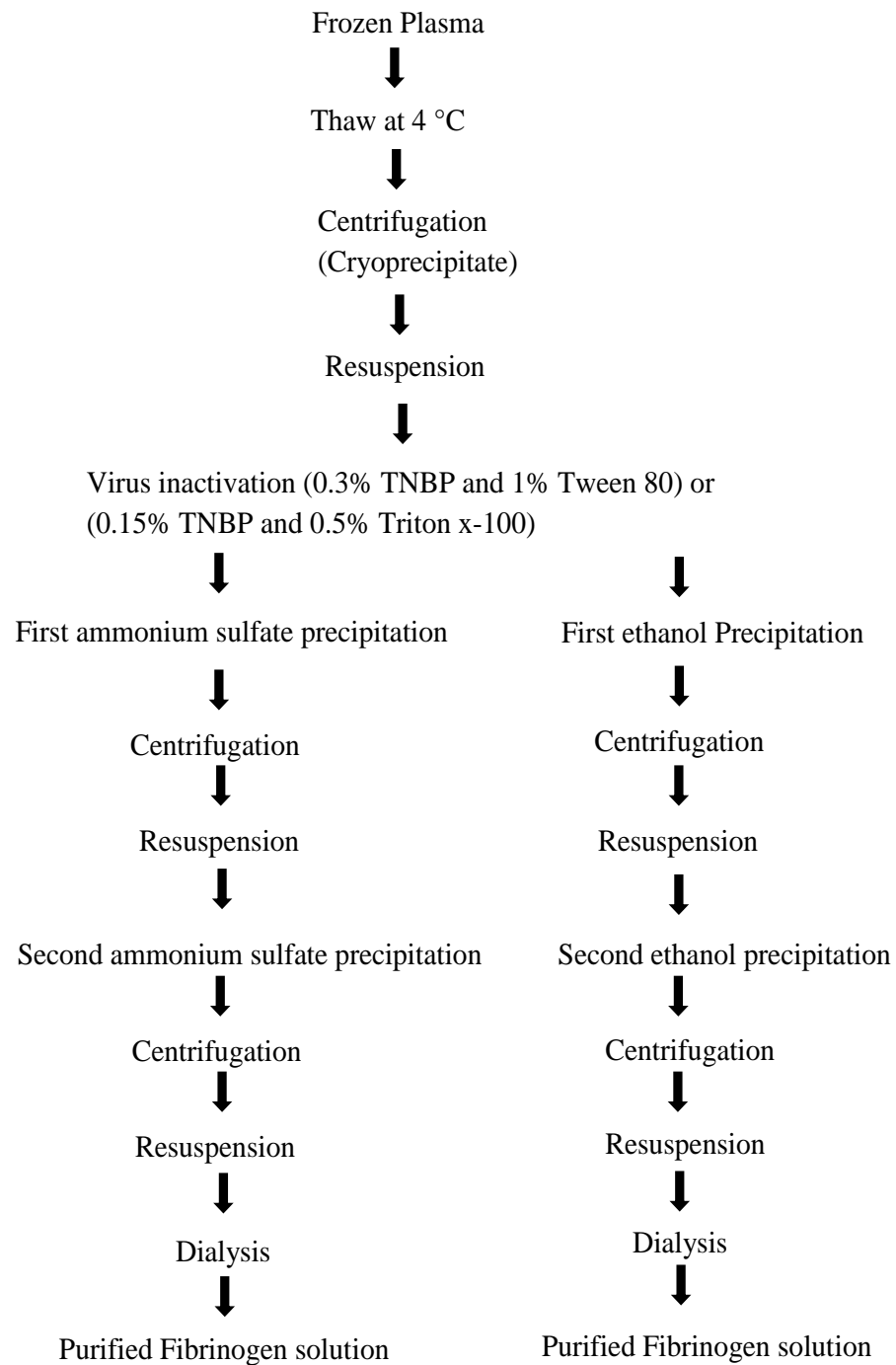
## **PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA**

We are employing a combination of physical and chemical precipitation methods to purify relatively large amount of fibrinogen from human plasma for the purpose of fibrin based bandages and expanding hemostatic foams. Virally inactivated fibrinogen was isolated from human plasma using cryoprecipitation followed by ethanol or ammonium sulfate precipitation. Fig 2 shows the major steps of the fibrinogen production procedure. The aim of this study was to evaluate the ability of ethanol and ammonium sulfate precipitation approaches to eliminate solvent detergent. We also estimated the amount of fibronectin, factor XIII A, factor XIII B, and  $\alpha_2$ -antiplasmin in plasma fibrinogen purified by the two different chemical precipitation methods.

## Materials and Methods

### Materials

All reagents of highest purity were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Human plasma was donated by the U.S. Army Materials Command (Fort Detrick, MD). Human plasma fibrinogen depleted of fibronectin, plasminogen, and von Willebrand Factor was purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant human thrombin (rFIIa) was purchased from ZymoGenetics, Inc. (Seattle, WA). Human FXIII was bought from Enzyme Research Laboratories. Human  $\alpha_2$ -AP was obtained from Haematologic Technologies Inc. (Essex Junction, VT). Anti-mouse fibronectin monoclonal IgG<sub>1</sub> antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human FXIIIB monoclonal antibody was purchased from Green Mountain Antibodies (Burlington, VT). Anti-human FXIIIA polyclonal antibody was bought from US Biologicals. Anti-human  $\alpha_2$ AP polyclonal antibody HRP was bought from US Biologicals. Anti-mouse IgG (whole molecule) peroxidase conjugate and anti-sheep IgG peroxidase conjugate were obtained from Sigma Chemical Company. Activated recombinant human factor XIII was purified in our lab. Thromboelastography (TEG) disposable cups and pins were bought from Haemoscope (Niles, IL).



**Figure 2: Fibrinogen purification procedure**

**Preparation of Cryoprecipitate**

Fifteen units of human plasma (12.65 liters) frozen at -80 °C were thawed slowly for two days at 4 °C. The resulting mixture was centrifuged in a Sorvall RC-5C Plus Superspeed Centrifuge (Kendro Laboratory, Newtown, CT) at 4000 rpm for 20 min at 4 °C. The supernatant was stored at -80 °C for future fibronectin purification. The fibrinogen-rich cryoprecipitate was re-suspended in a re-suspension buffer containing 20 mM tris-base, 55 mM sodium citrate, 27 mM lysine, PH 6.8. The solution was stirred overnight at room temp. Viral inactivation was carried out using two different types of solvent detergent. Part of the re-suspended cryoprecipitate was adjusted to 0.3% (V/V) TNBP and 1% (V/V) Tween 80 while the other portion was subjected to 0.15% (V/V) TNBP and 0.5% (V/V) Triton X-100 and the two solutions were stirred overnight at room temp.

**Ethanol Precipitation**

Ethanol precipitation method was conducted by slightly modifying a previously described method [52]. Briefly, the two types of solvent detergent treated solutions were cooled to ~4 °C on ice then adjusted to 10% ethanol. Samples were incubated on ice overnight then centrifuged in a Sorvall RC-5C Plus Superspeed Centrifuge at 4000 rpm at 4 °C for 20 min. The supernatant was discarded and the pellet was solubilized in re-suspension buffer. A second 10% ethanol precipitation step was carried out at ~4 °C for two hours on ice. The resulting pellet was solubilized in re-suspension buffer then dialyzed versus dialysis buffer containing 20 mM sodium citrate, 100 mM sodium

chloride, PH 7.4 at room temp. The dialyzed protein was collected and centrifuged for 20 min at 4000 rpm at 25 °C. Purified fibrinogen was stored in aliquots at - 80 °C.

### **Ammonium Sulfate Precipitation**

The virally-inactivated cryo solutions were chilled to ~4 °C on ice then adjusted to 1 M ammonium sulfate by adding a 4 M stock solution. Samples were incubated on ice overnight then centrifuged at 4000 rpm at 4 °C for 20 min. The precipitate was re-suspended in re-suspension buffer at room temp then chilled to ~4 °C on ice. A second ammonium sulfate precipitation was done and the pellet was solubilized in re-suspension buffer then dialyzed against dialysis buffer. The isolated protein was centrifuged for 20 min at 4000 rpm at 25 °C then frozen at -80 °C.

### **BCA Standard and Spectrophotometry**

Total protein concentration was determined by spectrophotometric measurement at 280 nm as well as BCA Standard procedure using bovine serum albumin as standard.

### **Evaluating Purified pdFI by SDS-PAGE**

The purity of the isolated fibrinogen was examined by sodium dodecylsulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Samples were analyzed on 4-12% NuPAGE Bis-Tris Mini Gels (Life Technologies, Carlsbad, CA) under reducing condition. Samples were treated with SDS sample buffer and reducing agent, both obtained from Invitrogen (Carlsbad, CA). After 10 minutes incubation at 74 °C, samples

were loaded onto the gel and run using 2-(N-morpholino) ethanesulfonic acid (MES) for one hour at 200 volts. Gels were stained with colloidal blue (Invitrogen, Carlsbad, CA).

### **Evaluating Clottability of pdFI by Thromboelastography**

Thromboelastography (TEG) Hemostasis system 5000 series (Haemoscope Corporation, Niles, IL) was used to evaluate the ability of purified fibrinogen to form a clot. pdFI (concentrations of 9 or 4.5 mg/ml) were placed in a TEG cup, which is kept at 37°C by the instrument. rFXIIIa (concentration of 0.36 mg/ml) was added. CaCl<sub>2</sub> (concentration of 12 mM) and Ringer solution (155 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>) were added followed immediately by rFIIa (concentration of 105.6 U/ml) to trigger clot formation. The TEG analyzer collected clot parameters such as the time to reach 2 mm clot strength (R), the time to reach 20 mm clot strength (K), and the maximum clot strength (MA) every five seconds for 30 minutes. The data was collected and analyzed in Microsoft Excel.

### **Estimating pdFXIIIA, pdFXIIIB, $\alpha_2$ -AP, and pdFN by Western Blot**

The contents of pdFXIIIA, pdFXIIIB,  $\alpha_2$ -AP, and pdFN on the isolated fibrinogen were estimated by quantitative western blot analysis. Samples were analyzed on 4-12% NuPAGE Bis-Tris Mini Gels (Life Technologies, Carlsbad, CA) under reducing or non-reducing condition. Gels were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) for 30 min at 25 volts. Blots were blocked in 50 ml 5% casein in TBST then incubated for 30 minutes with the following antibodies: (a) anti-human FXIIIA polyclonal antibody (US Biologicals, Swampscott, MA), (b) anti-

human FXIIIB monoclonal antibody (Green Mountain Antibodies, Burlington, VT), (c) anti-human  $\alpha_2$ AP polyclonal antibody HRP (US Biologicals), (d) anti-mouse fibronectin monoclonal IgG<sub>1</sub> antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blot developed with anti-human FXIIIA polyclonal antibody was subsequently detected with anti-sheep IgG peroxidase conjugate (Sigma). Blots developed with anti-human FXIIIB monoclonal antibody and anti-mouse fibronectin monoclonal IgG<sub>1</sub> antibody were probed with anti-mouse IgG peroxidase conjugate (Sigma). Blots were visualized with DAB/Metal concentrate and stable peroxide substrate buffer (Thermo Scientific, Rockford, IL) or chemiluminescence using Immun-Star HRP enhancer and peroxide buffer (Bio-Rad Laboratories, Hercules, CA). The quantity of pdFXIIIA, pdFXIIIB, pdFN, and  $\alpha_2$ -AP were determined from the immunoblot of each material using Imagej (National Institute of Health) or Adobe Photoshop Elements (Adobe Systems Incorporated).

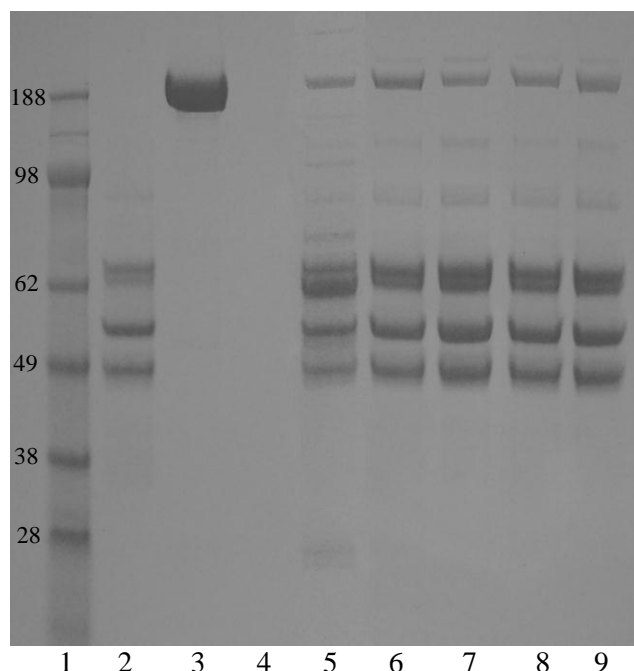
## Results

### Evaluating Purified pdFI by SDS-PAGE

SDS-PAGE analysis [Fig 3] conducted under reducing condition showed that pdFI purified by cryoprecipitation (lane 5), first ethanol precipitation (lane 6), second ethanol precipitation (lane 7), first ammonium sulfate precipitation (lane 8), and second ammonium sulfate precipitation (lane 9) all exhibit three distinct bands with apparent molecular weights of ~67, 54, 47 kDa likely corresponding to the well-established  $\alpha$ ,  $\beta$ , and  $\gamma$  chains of fibrinogen. Lanes 5, 6, 7, 8, and 9 also revealed a minor band at higher molecular weight of ~220 kDa characteristic of fibronectin monomer. pdFI isolated by



ethanol and ammonium sulfate precipitation contain two faint bands at ~80 and 120 kDa. In addition to the bands at ~80 and 120 kDa, pdFI purified by cryoprecipitation possesses additional faint bands.

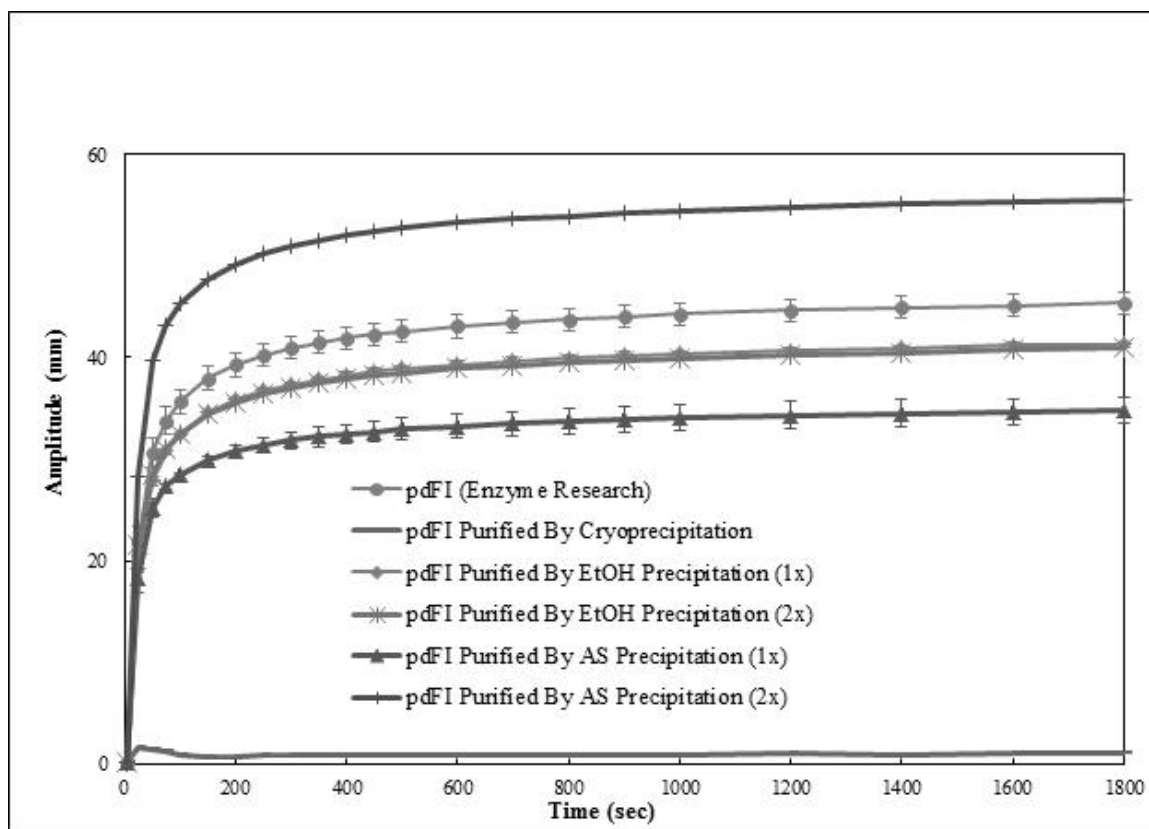


**Figure 3: Reduced gel evaluation of purified pdFI.**

Samples were analyzed on 4-12% SDS-PAGE and stained with colloidal blue. Lane 1 is molecular weight marker. Lane 2 is human fibrinogen (Enzyme Research). Lane 3 is human fibronectin (Enzyme Research). Lane 4 is blank. Lane 5 is pdFI purified by cryoprecipitation. Lanes 6 and 7 are pdFI purified by first and second AS precipitation, respectively. Lanes 8 and 9 are pdFI purified by first and second EtOH precipitation, respectively.

### **Evaluating Clottability of pdFI by Thromboelastography**

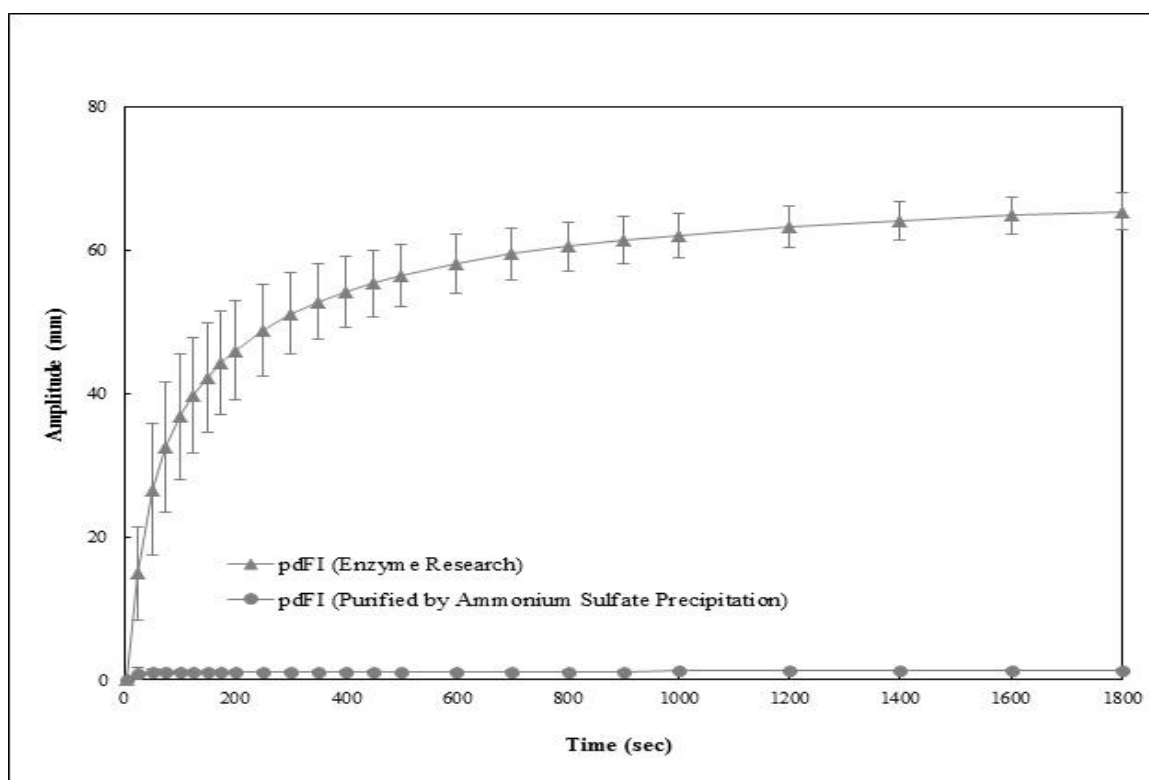
The viscoelastic and mechanical properties of clot formed by plasma fibrinogen purified by cryoprecipitation, ethanol, and ammonium sulfate precipitation were evaluated by thromboelastography [Fig 4]. It took 10 seconds to initiate clot formation after rFIIa was added to pdFI isolated by cryoprecipitation, ethanol, and ammonium sulfate precipitation [Table 6]. The maximal strength of clot developed by pdFI purified by the first ethanol precipitation (1x) was  $3267.70 \pm 9.05$  dynes/sec compared to  $3278.9 \pm 829.58$  dynes/sec for clot formed by pdFI purified by the second ethanol precipitation (2x). pdFI isolated by the second ammonium sulfate precipitation (2x) resulted in clot with maximal strength of  $5811.7 \pm 139.44$  dynes/sec whereas pdFI purified by the first ammonium sulfate precipitation (2x) formed clot with maximal strength of  $2483.75 \pm 167.09$  dynes/sec. Clot developed from pdFI purified by the second ammonium sulfate precipitated fibrinogen has the highest maximal strength ( $5811.7 \pm 139.44$ ). Clot formation was inhibited in the solvent detergent (TNBP and Triton X-100) treated pdFI purified by a single ammonium sulfate precipitation (Fig 5).



**Figure 4: Thromboelastography analysis of the clottability of isolated fibrinogen.** Change in maximal strength of clot developed by pdFI (4.5 mg/ml) activated by rFIIa (105.6 U/ml) was recorded over time.

**Table 6: Parameters of clot formed by purified fibrinogen**

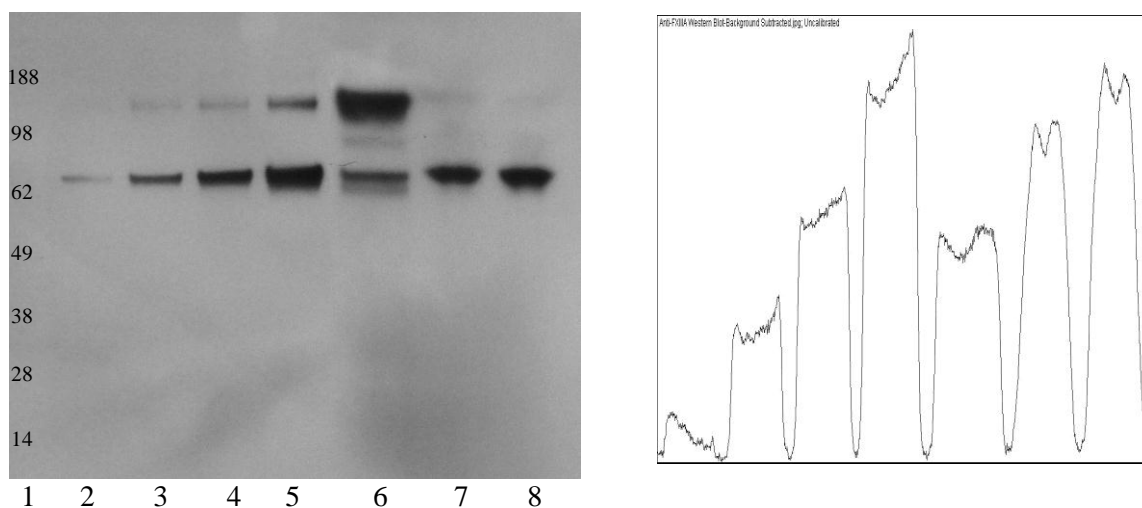
Protein	R (sec)	K (sec)	Angle (deg)	MA (mm)	G (d/sc)
pdFI (Enzyme Research)	10	50	84.2	43.3	3822.53
pdFI Purified By EtOH Precipitation (1x)	10	50	83.7	39.6	3267.70
pdFI Purified By AS Precipitation (1x)	10	50	82.9	33.2	2483.75
pdFI Purified By EtOH Precipitation (2x)	10	50	83.0	39.3	3278.9
pdFI Purified By AS Precipitation (2x)	10	50	85.5	53.8	5811.7
pdFI Purified By Cryoprecipitation	-2825	-225	-26.5	-2.2	-110.6



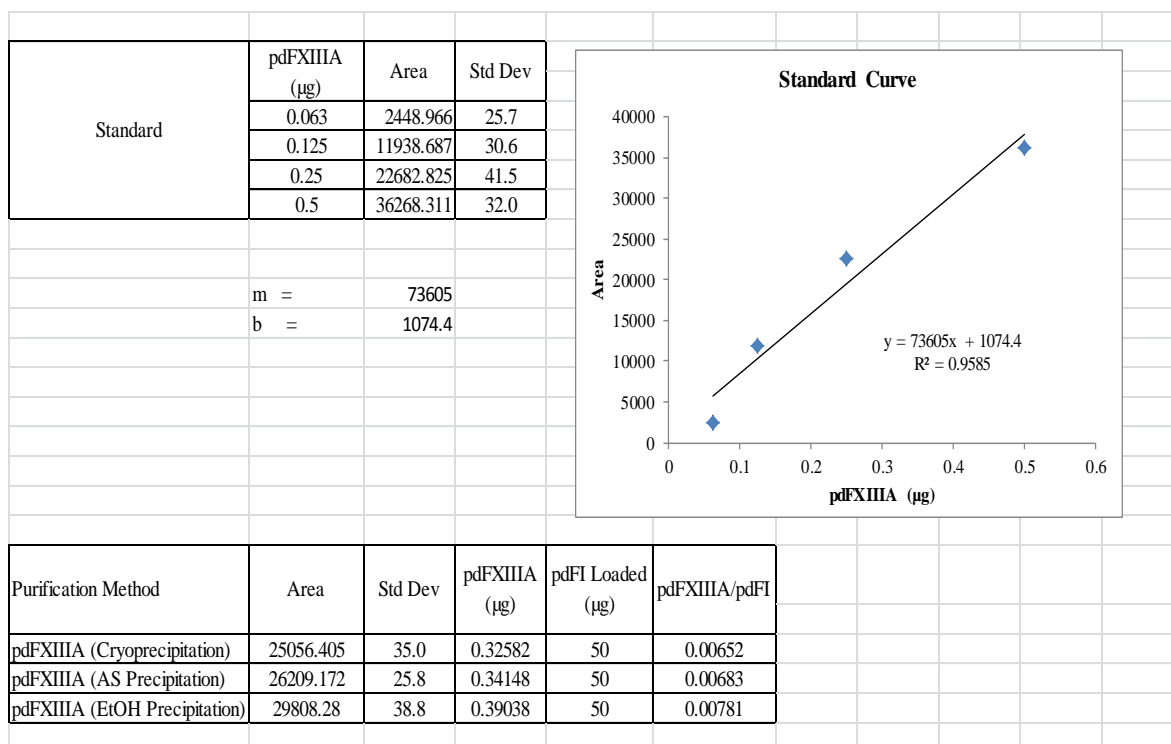
**Figure 5: Thromboelastography analysis of the clottability of fibrinogen purified by a single ammonium sulfate precipitation.** Clot was formed by treating plasma fibrinogen (9 mg/ml) with thrombin (105.6 U/ml).

#### Estimating Amounts of pdFXIIIA by Densitometry

Anti-human FXIIIA western blot analysis was conducted to estimate the percentage of FXIIIA in pdFI isolated by the three purification techniques [Fig 6]. pdFI purified by cryoprecipitation (lane 6), ammonium sulfate precipitation (lane 7), and ethanol precipitation (lane 8) show bands corresponding to the known molecular weight of pdFXIIIA. Using densitometry by Imagej, the percentages of FXIIIA in pdFI purified by cryoprecipitation, ethanol, and ammonium sulfate precipitation were 0.652%, 0.781%, and 0.683% respectively.

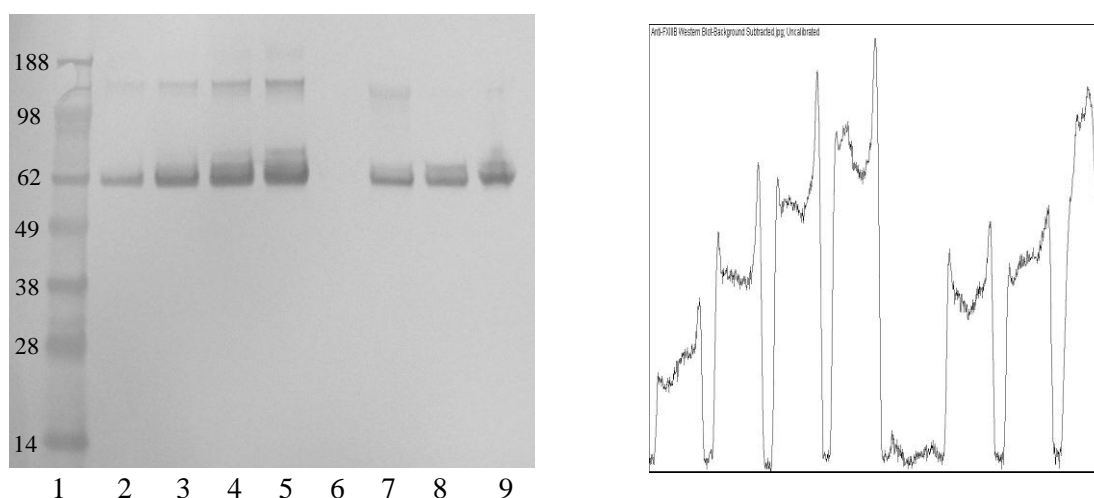


**Figure 6: Western blot and densitometry by Imagej analysis of pdFXIIIA.** Samples were analyzed under non-reducing condition then developed with anti-FXIIIA Pab and detected with anti-sheep IgG Peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.063, 0.125, 0.25, and 0.5 µg purified pdFXIIIA respectively. Lane 6 is pdFI purified by cryoprecipitation. Lane 7 is pdFI purified by ammonium sulfate precipitation. Lane 8 is pdFI purified by ethanol precipitation.

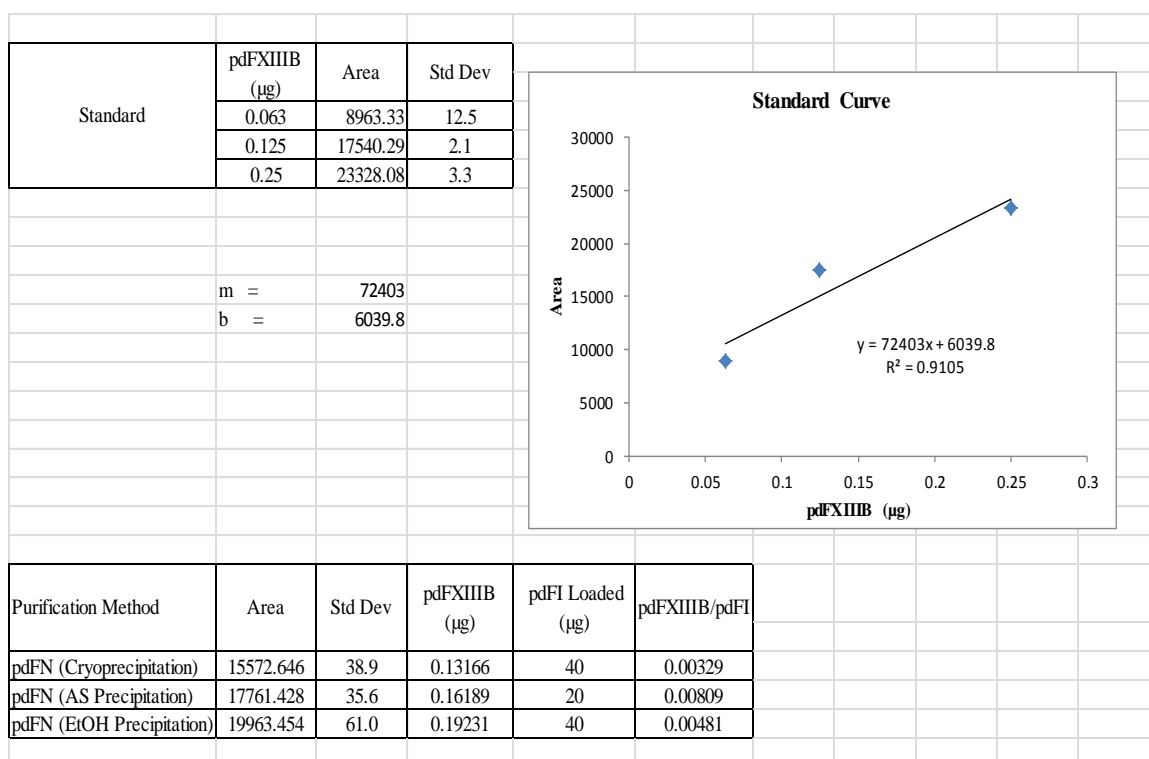


### Estimating Amounts of pdFXIIIB by Densitometry

The amounts of FXIIIB in pdFI precipitated by the three purification methods were calculated from the anti-human FXIIIB western blot analysis [Fig 7]. pdFI isolated by cryoprecipitation (lane 7), ammonium sulfate precipitation (lane 8), and ethanol precipitation (lane 9) show a band characteristic of the known molecular weight of pdFXIIIB. Densitometric measurement of the bands revealed that cryoprecipitation, ammonium sulfate, and ethanol precipitation pdFI contain 0.329%, 0.809%, and 0.481% respectively.

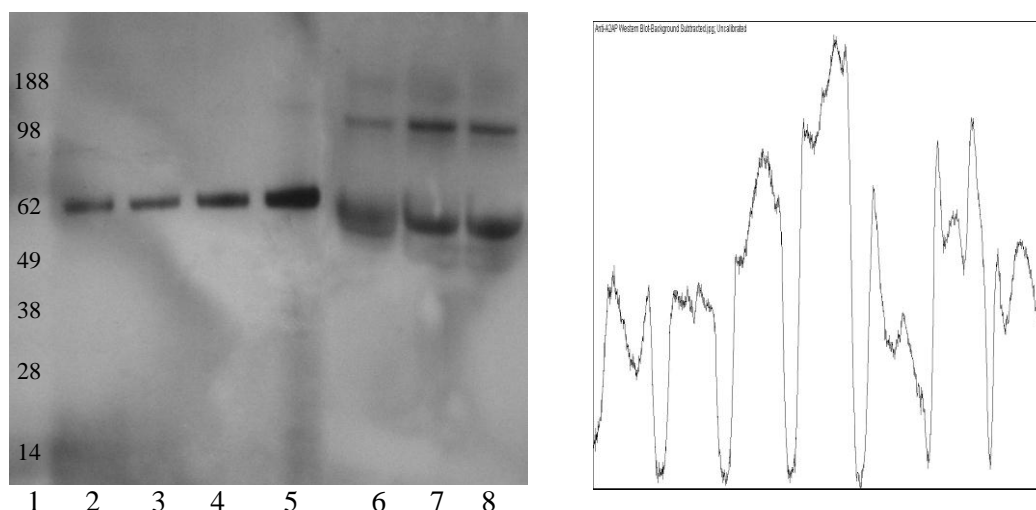


**Figure 7: Western blot and densitometry by Imagej analysis of pdFXIIIB.** Samples were analyzed under non-reducing condition and developed with anti-FXIIIB Mab then detected with anti-mouse IgG peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.063, 0.125, 0.25, and 0.5 µg purified pdFXIII respectively. Lane 6 is blank. Lane 7 is pdFI purified by cryoprecipitation. Lane 8 is pdFI purified by ammonium sulfate precipitation. Lane 9 is pdFI purified by ethanol precipitation.

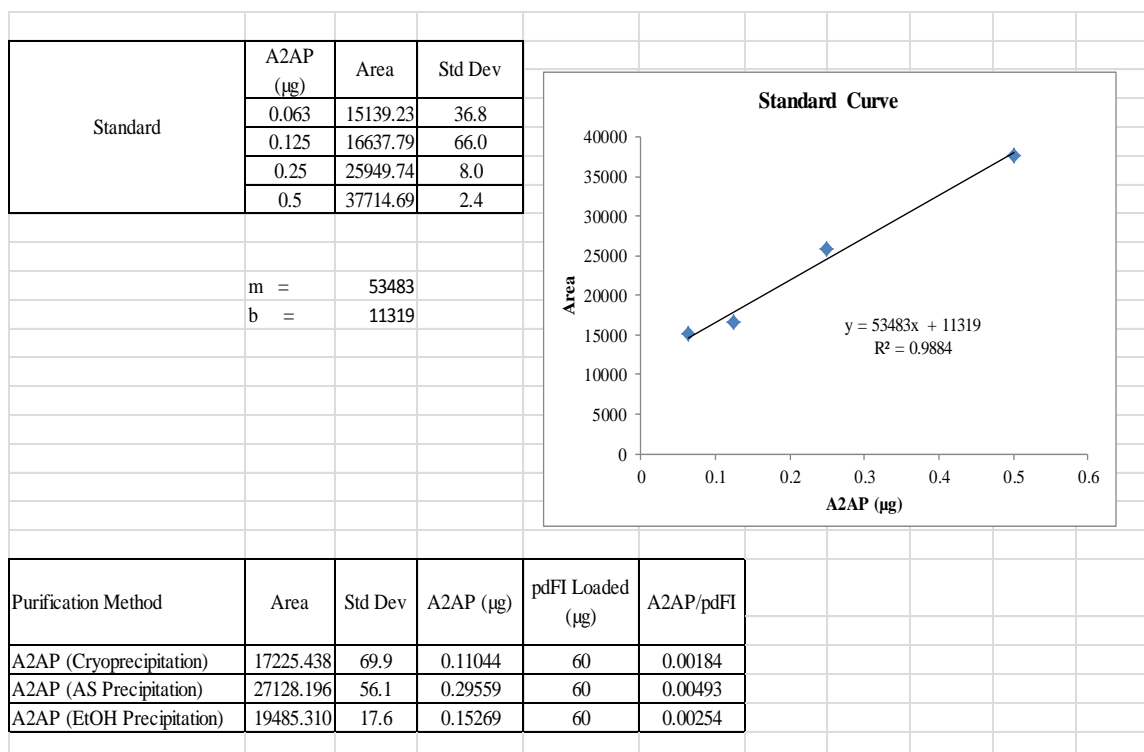


### Estimating Amounts of $\alpha_2$ -AP by Densitometry

The content of  $\alpha_2$ -AP in the purified pdFI was determined by anti-human  $\alpha_2$ AP western blot analysis [Fig 8]. pdFI isolated by cryoprecipitation (lane 6), ammonium sulfate (lane 7), and ethanol (lane 8) precipitation exhibit a minor band at ~62 kDa representing the molecular weight of  $\alpha_2$ -AP. By employing densitometry, pdFI purified by cryoprecipitation, ammonium sulfate, and ethanol precipitation contain 0.184%, 0.493%, and 0.254%  $\alpha_2$ -AP.



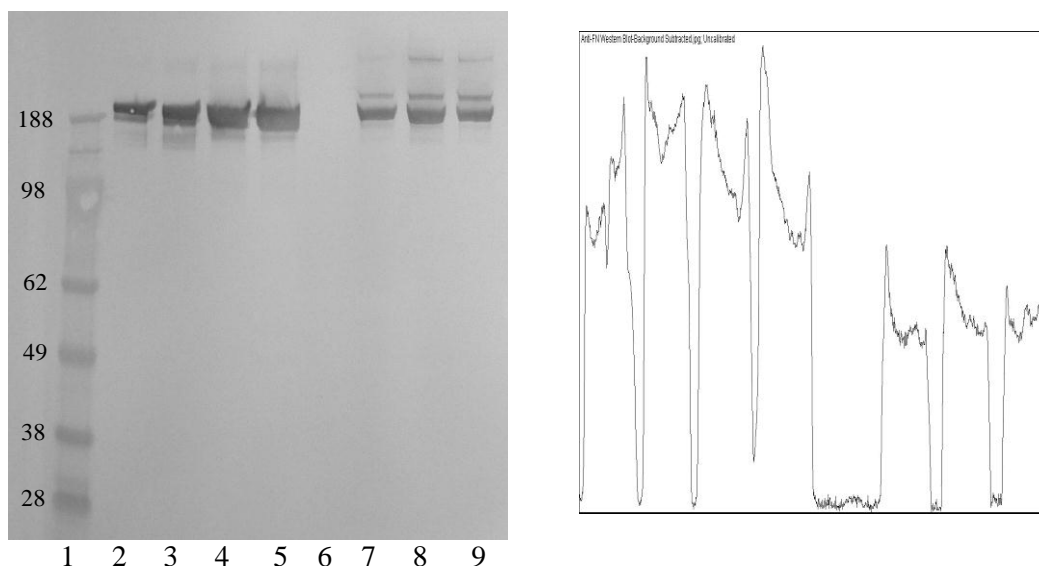
**Figure 8: Western blot and densitometry by Imagej analysis of  $\alpha_2$ -AP.** Samples were analyzed under reducing condition and developed with anti-  $\alpha_2$ -AP Pab HRP. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.013, 0.025, 0.05, and 0.1  $\mu$ g purified  $\alpha_2$ -AP respectively. Lane 6 is pdFI purified by cryoprecipitation. Lane 7 is pdFI purified by ammonium sulfate precipitation. Lane 8 is pdFI purified by ethanol precipitation.



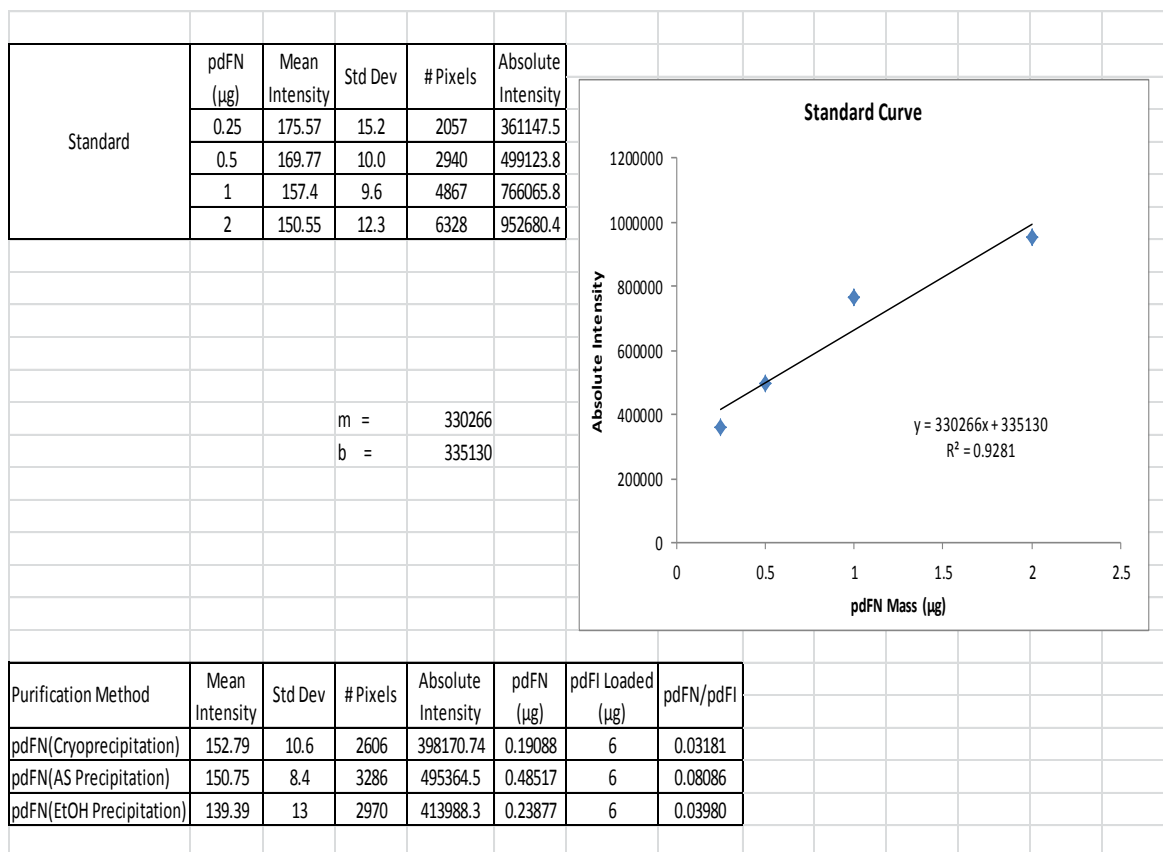


### Estimating Amounts of pdFN by Densitometry

Anti-human FN western blot analysis was done to estimate the quantity of FN purified with pdFI [Fig 9]. pdFI purified by cryoprecipitation (lane 7), ammonium sulfate (lane 8), and ethanol precipitation (lane 9) show bands at ~220 kDa corresponding to fibronectin monomer. Densitometry by Adobe Photoshop Elements was employed to calculate the percentage of FN in pdFI isolated by cryoprecipitation (3.18%), ammonium sulfate precipitation (8.09%), and ethanol precipitation (3.98%).



**Figure 9: Western blot and densitometry by Imagej analysis of pdFN.** Samples were analyzed under reducing condition and developed with anti-mouse fibronectin monoclonal IgG<sub>1</sub> then detected with anti-mouse IgG peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.25, 0.5, 1, and 2 µg purified pdFN respectively. Lane 6 is blank. Lane 7 is pdFI purified by cryoprecipitation. Lane 8 is pdFI purified by ammonium sulfate precipitation. Lane 9 is pdFI purified by ethanol precipitation.



## DISCUSSION

Various approaches have been developed to isolate fibrinogen from plasma sources. These methods include cryoprecipitation, centrifugation, ultrafiltration, chromatography, and chemical precipitation. We isolated a solvent detergent treated fibrinogen from human plasma using cryoprecipitation followed by ammonium sulfate or ethanol precipitation. Fibrinogen isolated by the three purification methods is highly pure. To determine the effectiveness of purified plasma fibrinogen to form clots, we measured clottability of fibrinogen using thromboelastography. Our study showed that clot formed from plasma fibrinogen produced by the repeated ammonium sulfate precipitation has the highest maximal strength.

Based on the purification method, fibrinogen removed from plasma contains various amounts of fibronectin, factor XIII,  $\alpha_2$ -antiplasmin, plasminogen, and other plasma proteins. We estimated the quantities of fibronectin, factor FXIIIA, factor FXIIIB, and  $\alpha_2$ -AP in the plasma fibrinogen isolated by three purification procedures. Plasma fibrinogen obtained by ammonium sulfate precipitation was found to contain increased ratios of FXIIIA, FXIIIB, FN, and  $\alpha_2$ -AP to fibrinogen than the starting cryoprecipitate. Plasma fibrinogen from ethanol precipitation also contains higher ratios of FXIIIA, FXIIIB,  $\alpha_2$ -AP, and FN to fibrinogen than the original cryoprecipitate. This indicates that chemical precipitation may preserve the binding of FXIIIA, FXIIIB, FN, and  $\alpha_2$ -AP to fibrinogen during the purification process.

Viral inactivation was achieved by using two different types of solvent detergent mixtures: (1) 0.3% TNBP and 1% Tween 80, (2) 0.15% TNBP and 0.5% Triton X-100. Since the presence of solvent detergent inhibits clot formation, we utilized thromboelastography to evaluate the effectiveness of ethanol and ammonium sulfate precipitation to remove the solvent detergent. While purifying a solvent detergent treated fibrinogen from human plasma, Burnouf-Radosevich et al [52] applied a second ethanol precipitations to further remove the solvent detergent. However, we showed that clots formed from fibrinogen isolated by first and second ethanol precipitation have similar maximal strength. This indicates that a single ethanol precipitation step is capable of eliminating the solvent detergent. A single ammonium sulfate precipitation partially removed the mixture 0.3% TNBP and 1% Tween 80 but not the mixture 0.15% TNBP and 0.5% Triton x-100. Thus, a second ammonium sulfate precipitation is required for the complete elimination of the solvent detergent mixture TNBP and Triton X-100.

Together the data presented in this thesis indicate that the combination of cryoprecipitation and successive ammonium sulfate precipitations results in the fibrinogen with the strongest clot forming activity. The bases for the increased strength has not been determined but is likely due to the increased ratios of associated proteins, particularly fibronectin in the ammonium sulfate derived material.

## REFERENCES

1. Galina Tsurupa; Latchezar Tsonev; Leonid Medved. Structural Organization of the Fibrin(ogen)  $\alpha$ C-Domain. *Biochemistry* 41: 6449-6459 (2002).
2. Henschen A; Lottspeich F; Kehl M; Southan C. Covalent structure of fibrinogen *Ann N Y Acad Sci* 408: 28-43 (1983).
3. Privalov PL; Medved LV. *J Mol Biol* 159: 665-683 (1982).
4. Medved LV; Litvinovich S; Ugarova T; Matsuka Y; Ingham K. *Biochemistry* 36: 4685-4693 (1997).
5. Medved LV; Gorkun OV; Privalov PL. *FEBS Lett* 160: 291-295 (1983).
6. Doolittle RF; Annu Rev. *Biochem* 53:195-229 (1984).
7. Brown JH; Volkmann N; Jun G; Henschen-Edman AH; Cohen C. *Proc Natl Acad Sci USA* 97: 85-90 (2000).
8. Weisel JW; Stauffacher CV; Bullitt E; Cohen C. *Science* 230: 1388-1391 (1985).
9. McKee P.A; Rogers L.A; Marler E; Hill R.L. *Arch. Biochem. Biophys.* 1966; 116, 271-279.
10. Doolittle RF. *Thromb Haemost* (Bloom AL; Thomas DP, eds) 163-197 (1981), Churchill Living-stone, Edinburgh.
11. Townsend RR; Hilliker E; Li YT; Laine RA; Bell WR; Lee YC. *J Biol Chem* 257: 9704-9710 (1982).
12. Finlayson JS; Mosesson MW. Heterogeneity of human fibrinogen. *Biochemistry* 2: 42-46 (1963).
13. Mosesson MW; Finlayson JS; Umfleet RA. Human fibrinogen heterogeneities, III: identification of  $\gamma$ -chain variants. *J Biol Chem.* 247: 5223-5227 (1972).
14. Wolfenstein-Todel C; Mosesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal gamma chain variant ( $\gamma'$ ). *Proc Natl Acad Sci USA* 77: 5069-5073 (1980).
15. Chung DW; Davie EW.  $\gamma$  and  $\gamma'$  chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry* 23:4232-4236 (1984).
16. Farrell DH; Mulvihill ER; Huang SM; Chung DW; Davie EW. Recombinant human fibrinogen and sulfation of the gamma chain. *Biochemistry* 30:9414-9420 (1991).
17. Stathakis N.E.; M.W. Mosesson. Interactions among heparin, cold-insoluble globulin, and fibrinogen in formation of the heparin precipitable fraction of plasma. *J. Clin. Invest.* 60: 855-865 (1977).
18. Sahni A.; T. Odrlic; C.W. Francis. Binding of basic fibroblast growth factors to fibrinogen and fibrin. *J. Biol. Chem.* 273: 7554-7559 (1998).
19. Loscalzo J.; M. Weinfeld; G.M. Fless; A.M. Scanu. Lipoprotein(a), fibrin binding, and plasminogen activation. *Arteriosclerosis* 10: 240-245 (1990).
20. Hervio L.; V. Durlach; A. Girard-Globa; E. Angles-Cano. Multiple binding with identical linkage: a mechanism that explains the effect of lipoprotein(a) on fibrinolysis. *Biochemistry* 34: 13353-13358 (1995).

21. Meh D.A.; K.R. Siebenlist; M.W. Mosesson. Identification and characterization of the thrombin binding sites on fibrin. *J. Biol. Chem.* 271: 23121–23125 (1996).
22. Bok R.A.; W.F. Mangel. Quantitative characterization of the binding of plasminogen to intact fibrin clots, lysine-sepharose, and fibrin cleaved by plasmin. *Biochemistry* 24: 3279–3286 (1985).
23. Spraggon G; Everse SJ; Doolittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature* 389:455-462 (1997).
24. Weisel JW; Francis CW; Nagaswami C; Marder VJ. Determination of the topology of factor XIIIa induced fibrin gamma-chain cross-links by electron microscopy of ligated fragments. *J Biol Chem.* 268:26618-26624 (1993).
25. Purves L; Purves M; Brandt W. Cleavage of fibrin-derived D-dimer into monomers by endopeptidase from puff adder venom (*Bitis arietans*) acting at cross-linked sites of the gamma-chain: sequence of carboxy-terminal cyanogen bromide gamma-chain fragments. *Biochemistry* 26: 4640-4646 (1987).
26. Tamaki T.; H. Aoki. Cross-linking of  $\alpha$ 2-plasmin inhibitor and fibronectin to fibrin by fibrin-stabilizing factor. *Biochim. Biophys. Acta* 661: 280–286 (1981).
27. Michael S. Kostelansky; Karim C. Lounes; Li Fang Ping; Sarah K. Dickerson; Oleg V. Gorkun; Susan T. Lord. Calcium-Binding Site  $\beta$ 2, Adjacent to the 'b' Polymerization Site, Modulates Lateral Aggregation of Protofibrils during Fibrin Polymerization. *Biochemistry* 34, 2475-2483 (2004).
28. Fornace A.J. Jr.; Cummings D.E.; Comeau C.M.; Kant J.A.; Crabtree G.R. *J. Biol. Chem.* 259: 12826 (1984).
29. Hortin G.L. *Biochem. Int.* 19: 1355 (1989).
30. Heldin P.; Hessel B.; Humble E.; Blomback B.; Engstrom L. *Thromb. Res.* 47: 93 (1987).
31. Dang C.V.; Shin C.K.; Bell W.R.; Nagaswami C.; Weisel J.W. *J. Biol. Chem.* 264: 5104 (1989).
32. Francis C.W.; Keele E.M.; Marder V.J. *Biochim. Biophys. Acta* 797: 328 (1984).
33. De Maat MPM; Verschuur M; Fibrinogen heterogeneity: inherited and non-inherited. *Curr Opin Hematol* 12: 377-83 (2005).
34. Henschen A.H. *Thromb. Haemost.* 70: 42 (1993).
35. Olexa SA; Budzynski AZ. *Proc. Natl. Acad. Sci. U.S.A.* 77: 1374-8 (1980).
36. Everse SJ; Spraggon G; Veerapandian L; Riley M; Doolittle RF. *Biochemistry* 37: 8637-8642 (1998).
37. Mosesson MW; Siebenlist KR; Hainfeld JF; Wall JS. The covalent structure of factor XIIIa crosslinked fibrinogen fibrils. *J Struct Biol* 115: 88–101(1995).
38. Siebenlist KR; Meh D; Mosesson MW. Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thromb Haemost* 86: 1221–8 (2001).
39. McKee PA; Mattock P; Hill RL. Subunit structure of human fibrinogen, soluble fibrin, and cross-linked insoluble fibrin. *Proc Natl Acad Sci USA* 66: 738–44 (1970).
40. Kanaide H; Shainoff JR. Cross-linking of fibrinogen and fibrin by fibrin-stabilizing factor (factor XIIIa). *J Lab Clin Med* 85: 574–97 (1975).

41. Doolittle RF; Chen R; Lau F. Hybrid fibrin: proof of the intermolecular nature of c-c-crosslinking units. *Biochem Biophys Res Commun* 44: 94–100 (1971).
42. Chen R; Doolittle RF. c-c-Cross-linking sites in human and bovine fibrin. *Biochemistry* 10: 4486–91(1971).
43. Siebenlist KR; Mosesson MW. Evidence for intramolecular crosslinked  $\alpha$ c chain heterodimers in plasma fibrinogen. *Biochemistry* 35: 5817–21(1996).
44. Wan H.L.; S T. Huang; D M. Floyd; D H. Sierra. Is the amount of fibrinogen in cryoprecipitate is adequate for fibrin glue? Introducing an improved recycle cryoprecipitate method. *Transfusion* 29: 41 (1989).
45. Park M.S.; C.I. Cha. Biochemical aspects of autologous fibrin glue derived from ammonium sulfate precipitation. *Laryngoscope* 103(2): 193-6 (1993).
46. Blombaeck B.; Blombaeck M. Purification of human and bovine fibrinogen. *Arkiv. Kemi.* 10: 415–443 (1956).
47. Straughn W.; Wagner R.H. A simple method for preparing fibrinogen. *Thromb. Diath. Haematol.* 16: 198–206 (1966).
48. Keckwick R.A.; Mackay M.E.; Nance M.H. The purification of human fibrinogen. *Biochem. J.* 60: 671–678 (1955).
49. Laki K. The polymerization of proteins: the action of thrombin on fibrinogen. *Arch. Biochem. Biophys.* 32: 317–324 (1951).
50. Chuansumrit A; Isarangkura P; Chantanakajornfung A; Kuhathong K; Pintadit P; Jitpraphai C; Hathirat P; Nuchprayoon C. The efficacy and safety of lyophilized cryoprecipitate in hemophilia A. *J Med Assoc Thai* 82: S69–S73 (1999).
51. Hornsey VS; Krailadsiri P; MacDonald S; Seghatchian J; Williamson LM; Prowse CV. Coagulation factor content of cryoprecipitate prepared from methylene blue plus light virusinactivated plasma. *Br J Haematol* 109:665–670 (2000).
52. M. Burnouf-Radosevich, T. Burnouf, J. J. Huart. Biochemical and physical properties of a solvent-detergent-treated fibrin glue. *Vox Sang* 58: 77-84 (1990).
53. Bernard Horowitz; Richard Bonomo; Alfred M. Prince; Sing N. Chin; Betsy Brotman; Richard W. Shulman. Solvent/Detergent-Treated Plasma: A Virus-Inactivated Substitute for Fresh Frozen Plasma. *Blood* 79: 826-831 (1992).
54. Muszbek L; Yee VC; Hevessy Z. *Thromb. Res.* 94: 271 (1999).
55. Greenberg CS; Sane DC; Lai T; Colman RW; Marder VJ; Clowes AW; George JN; Goldhaber SZ. Eds; Lippincott Williams; Wilkins: Philadelphia, *Hemostasis and Thrombosis*, 5th edn. pp.153-81 (2006).
56. Brian T; Turner Jr; Muriel C. Maurer. *Biochemistry* 41: 7947-7954 (2002).
57. Bishop PD; Teller DC; Smith RA; Lasser GW; Gilbert T; Seale RL. Expression, Purification, and characterization of human factor XIII in *Saccharomyces cerevisiae*. *Biochemistry* 29: 861-9 (1990).
58. Carrell NA; Erickson HP; McDonagh J. Electron microscopy and hydrodynamic properties of factor XIII subunits. *J Biol Chem* 264: 551-6 (1989).
59. Jan McDonagh. Structure and function of factor XIII. *Hemost and Thromb*, 1994.
60. Schwartz ML; Pizzo SV; Hill RL; McKee PA. Human factor XIII from plasma and platelets. Molecular weights, subunit structures, proteolytic activation, and cross-linking of fibrinogen and fibrin. *J Biol Chem* 248:1395 (1973).

61. Mary A, Achyuthan KE, Greenberg CS. B-chains prevent the proteolytic inactivation of the a-chains of plasma factor XIII. *Biochim Biophys Acta* 966: 328 (1988).
62. Katona E; Haramura G; Karpati L; Fachel J; Muszbek L. A simple, quick one-step ELISA assay for the determination of complex plasma factor XIII (A2B2). *Thromb Haemost* 83: 268–73 (2000).
63. Ichinose A.; McMullen B. A.; Fujikawa K.; Davie E. W. Amino acid sequence of the B subunit of human Factor XIII, a protein composed of ten repetitive segments. *Biochemistry* 25: 4633–4638 (1986).
64. Ichinose A.; Bottenus R. E.; Davie E. W. Structure of transglutaminase. *J. Biol. Chem.* 265: 13411–13414 (1990).
65. Nagy JA, Kradin RL, McDonagh J. Biosynthesis of factor XIII A and B subunits. *Adv Exp Med Biol.* 231: 29-49 (1988).
66. Weisberg L.J.; Shiu DT; Conkling PR; Shuman MA. Identification of normal human peripheral blood monocytes and liver as sites of synthesis of coagulation factor XIII a-chain. *Blood* 70: 579-582 (1987).
67. A' da'ny R; Nemes Z; Muszbek L. Characterization of factor XIII containing-macrophages in lymph nodes with Hodgkin's disease. *Br J Cancer* 55: 421-426 (1987).
68. Lozier J.; Takahashi N.; Putnam F. W. Complete amino acid sequence of human plasma b2-glycoprotein I. *Proc. Natl. Acad. Sci. U.S.A.* 81: 3640–3644 (1984).
69. Weiss M.S.; Metzner HJ; Hilgenfeld R. Two non-proline cis peptide bonds may be important for factor XIII function. *FEBS Lett.* 423: 291-296 (1998).
70. Schwartz M.I.; Pizzo SV; Hill RL; McKee PA. The subunit structure of human plasma and platelet factor XIII (fibrin-stabilizing factor). *J Biol Chem.* 246: 5851-5854 (1971).
71. Henriksson T.H.; Becker S; McDonagh J: Identification of intracellular factor XIII in human monocytes and macrophages. *J Clin Invest* 76:528 (1985).
72. Kiesselbach T.H.; Wagner RH: Demonstration of factor XIII in human megakaryocytes by a fluorescent antibody technique. *Ann NY Acad Sci* 202: 318 (1972).
73. Yee V.C.; Pedersen LC; Bishop PD; Stenkamp RE; Teller DC. Structural evidence that the activation peptide is not released upon thrombin cleavage of factor XIII. *Thromb Res* 78:389-97 (1995).
74. Grundman U; Amann E; Zettlmeissl G; Kupper HA: Characterization of a cDNA coding for human factor XIIIa. *Proc Natl Acad Sci USA* 83:8024 (1986).
75. Schroeder V; Vuissoz JM; Caflisch A; Kohler HP. Factor XIII activation peptide is released into plasma upon cleavage by thrombin and showed a different structure compared to its bound form. *Thromb Haemost* 97: 890-8 (1993).
76. Hornyak T.J.; Shafer JA. Role of calcium ion in the generation of factor XIII activity. *Biochemistry* 30: 6175–82 (1991).
77. Lewis S.D.; Janus TJ; Lorand L; Shafer JA. Regulation of formation of factor XIIIa by its fibrin substrates. *Biochemistry* 24: 6772–7 (1985).
78. Sakata Y; Aoki N. Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J Clin Invest.* 65: 290-297 (1980).



79. Kimura S; Aoki N. Cross-linking site in fibrinogen for  $\alpha_2$ -plasmin inhibitor. *J Biol Chem.* 261: 15591-15595 (1986).
80. Kimura S; Tamaki T; Aoki N. Acceleration of fibrinolysis by the N-terminal peptide of  $\alpha_2$ -plasmin inhibitor. *Blood* 66: 157-160 (1985).
81. Cottrell B.A.; Strong DD; Watt KW; Doolittle RF. Amino acid sequence studies on the  $\alpha$ -chain of human fibrinogen: exact location of cross-linking acceptor sites. *Biochemistry* 18: 5405-5410 (1979).
82. Fretto L.J.; Ferguson EW; Steinman HM; McKee PA. Localization of the  $\alpha$ -chain cross-link acceptor sites of human fibrin. *J Biol Chem.* 253: 2184-2195 (1978).
83. Matsuka Y.V.; Medved LV; Migliorini MM; Ingham KC. Factor XIIIa-catalyzed cross-linking of recombinant  $\alpha$ C fragments of human fibrinogen. *Biochemistry* 35: 5810-5816 (1996).
84. Mosher D.F.; Schad PE; Vann JM. Cross-linking of collagen and fibronectin by factor XIIIa: localization of participating glutamyl residues to a tryptic fragment of fibronectin. *J Biol Chem.* 255: 1181-1188 (1980).
85. Procyk R; Adamson L; Block M; Blomback B. Factor XIII catalyzed formation of fibrinogen-fibronectin oligomers: a thiol enhanced process. *Thromb Res.* 40: 833-852 (1985).
86. Mosher D.F.; Schad PE. Cross-linking of fibronectin to collagen by blood coagulation factor XIIIa. *J Clin Invest.* 64: 781-787 (1979).
87. Hada M; Kaminski M; Bockenstedt P; McDonagh J. Covalent crosslinking of von Willebrand factor to fibrin. *Blood* 68: 95-101(1986).
88. Bockenstedt P; McDonagh J; Handin RI. Binding and covalent cross-linking of purified von Willebrand factor to native monomeric collagen. *J Clin Invest.* 78: 551-556 (1986).
89. Sane D.C.; Moser TL; Pippen AM; Parker CJ; Achyuthan KE; Greenberg CS. Vitronectin is a substrate for transglutaminases. *Biochem Biophys Res Commun.* 157:115-120 (1988).
90. Skorstengaard K; Halkier T; Hojrup P; Mosher D. Sequence location of a putative transglutaminase cross-linking site in human vitronectin. *FEBS Lett.* 262: 269-274 (1990).
91. Francis R.T.; McDonagh J; Mann KG. Factor V is a substrate for the transamidase factor XIIIa. *J Biol Chem.* 261: 9787-9792 (1986).
92. Huh M.M.; Schick BP; Schick PK; Colman RW. Covalent crosslinking of human coagulation factor V by activated factor XIII from guinea pig megakaryocytes and human plasma. *Blood* 71: 1693-1702 (1988).
93. Bale M.D.; Westrick LG; Mosher DF. Incorporation of hrombospondin into fibrin clots. *J Biol Chem.* 260: 7502-7508 (1985).
94. Mui P.T.; Ganguly P. Cross-linking of actin and fibrin by fibrin-stabilizing factor. *Am J Physiol.* 233: H346-H349 (1977).
95. Cohen I; Blankenberg TA; Borden D; Kahn DR; Veis A. Factor XIIIa-catalyzed cross-linking of platelet and muscle actin: regulation by nucleotides. *Biochim Biophys Acta.* 628: 365-375 (1980).

96. Valnickova Z; Enghild JJ. Human procarboxypeptidase U, or thrombin-activable fibrinolysis inhibitor, is a substrate for transglutaminases: evidence for transglutaminase-catalyzed cross-linking to fibrin. *J Biol Chem.* 273: 27220-27224 (1998).
97. Ritchie H; Lawrie LC; Crombie PW; Mosesson MW; Booth NA. Cross-linking of plasminogen activator inhibitor 2 and alpha 2-antiplasmin to fibrin(ogen). *J Biol Chem.* 275: 24915-24920 (2000).
98. Ritchie H; Lawrie LC; Mosesson MW; Booth NA. Characterization of crosslinking sites in fibrinogen for plasminogen activator inhibitor 2 (PAI-2). *Ann N Y Acad Sci.* 936: 215-218 (2001).
99. Mosesson M.W. Fibrinogen  $\gamma$  chain functions. *Thromb Haemost* 1:231-238 (2002).
100. Greenberg C.S.; Miraglia CC. The effect of fibrin polymers on thrombin-catalyzed plasma factor XIIIa formation. *Blood* 66: 466-9 (1985).
101. Greenberg C.S.; Achyuthan KE; Rajagopalan S; Pizzo SV. Characterization of the fibrin polymer structure that accelerates thrombin cleavage of plasma factor XIII. *Arch Biochem Biophys* 262: 142-8 (1988).
102. Naski M.C.; Lorand L; Shafer JA. Characterization of the kinetic pathway for fibrin promotion of alpha-thrombin-catalyzed activation of plasma factor XIII. *Biochemistry* 30: 934-41 (1991).
103. Janus TJ; Lewis SD; Lorand L; Shafer JA. Promotion of thrombin catalyzed activation of factor XIII by fibrinogen. *Biochemistry* 22: 6269-72 (1983).
104. Hornyak TJ; Shafer JA. Interactions of factor XIII with fibrin as substrate and cofactor. *Biochemistry* 31: 423-9 (1992).
105. Greenberg CS; Achyuthan KE; Fenton JW II. Factor XIIIa formation promoted by complexing of alpha-thrombin, fibrin, and plasma factor XIII. *Blood* 69: 867-71 (1987).
106. Siebenlist KR; Meh DA; Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing  $\gamma'$  chains. *Biochemistry* 35:10448-53 (1996).
107. Lynch GW; Pfueller SL. Thrombins-independent activation of platelet factor XIII by endogenous platelet acid protease. *Thromb Haemost* 59:372-377 (1988).
108. Francis RT; McDonagh J; Mann KG. *J. Biol. Chem* 26: 9787(1986).
109. Jansen PH; Lorand L; Ebbesen P; Gliemann J. *Eur. J.Biochem* 214:141 (1993).
110. Bendixen E; Borth W; Harpel PC. *J. Biol. Chem* 268:21962 (1993).
111. Borth W; Chang V; Bishop P; Harpel PC. *J. Biol. Chem* 266: 18149 (1991).
112. Kangsadalampai S; Chelvanayagam G; Baker RT; Yenchitsomanus P; Pungamritt P; Mahasandana C; Board PG. A novel Asn344 deletion in the core domain of coagulation factor XIII A subunit: Its effects on protein structure and function. *Blood* 92: 481-7 (1998).
113. Koyama T; Koike Y; Toyota S; Miyagi F; Suzuki N; Aoki N. Different NH<sub>2</sub>-terminal form with 12 additional residues of alpha 2-plasmin inhibitor from human plasma and culture media of Hep G2 cells. *Biochem Biophys Res Commun* 200: 417-22 (1994).

114. Bangert K; Johnsen AH; Christensen U; Thorsen S. Different N-terminal forms of alpha 2-plasmin inhibitor in human plasma. *Biochem J* 291 (Pt 2): 623–5 (1993).
115. Coughlin PB. Antiplasmin: the forgotten serpin? *FEBS J* 272: 4852–7 (2005).
116. Aoki N. Genetic abnormalities of the fibrinolytic system. *Semin Thromb Hemost* 10: 42–50 (1984).
117. Collen D; Wiman B. Turnover of antiplasmin, the fast-acting plasmin inhibitor of plasma. *Blood* 53: 313–24 (1979).
118. Lee KN; Jackson KW; Christiansen VJ; Chung KH; McKee PA. A novel plasma proteinase potentiates  $\alpha_2$ -antiplasmin inhibition of fibrin digestion. *Blood* 103: 3783–3788 (2004).
119. Hirose S.; Nakamura Y.; Miura O.; Sumi, Y.; Aoki, N. *Proc.Natl. Acad. Sci. U.S.A.* 85: 6836 (1988).
120. Sumi, Y; Nakamura Y; Aoki N; Sakai M; Muramatsu M. Structure of the carboxyl-terminal half of human R2-plasmin inhibitor deduced from that of cDNA. *J. Biochem.* 100: 1399–13402 (1986).
121. Pierschbacher MD; Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small fragments of the molecule. *Nature* 309: 30–33 (1984).
122. Law R H; Sofian T; Kan WT; Horvath AJ; Hitchen CR; Langendorf CG; Buckle AM; Whisstock JC; Coughlin PB. X-ray crystal structure of the fibrinolysis inhibitor R2-antiplasmin. *Blood* 111: 2049–2052 (2008).
123. Sasaki T; Morita T; Iwanaga S. Identification of the plasminogen-binding site of human R2-plasmin inhibitor. *J. Biochem.* 99: 1699–1705 (1986).
124. Frank PS; Douglas JT; Locher M Llinas M; Schaller J. Structural/functional characterization of the R2-plasmin inhibitor C-terminal peptide. *Biochemistry* 42: 1078–1085 (2003).
125. Aoki N; Harpel PC. *Semin. Thromb. Hemostasis* 10: 24–41 (1984).
126. Moroi M; Aoki N. *J. Biol. Chem.* 261: 5956–5965 (1976).
127. Aoki N; Moroi M; Tachiya K. *Thromb. Haemostasis* 39: 22–31 (1978).
128. Ichinose A; Koide T; Aoki N. *Thromb. Res.* 33: 401–407 (1984).
129. Sakata Y; Aoki N. *J. Clin. Invest.* 65: 290–297 (1980).
130. Sakata Y; Aoki N. *J. Clin. Invest.* 69: 536–542 (1982).
131. Tamaki T; Aoki N. *J. Biol. Chem.* 267: 14767–14772 (1982).
132. Wiman B; Boman L; Collen D. *Eur. J. Biochem* 87: 143 (1978).
133. Sasaki T; Morita T; Iwanaga S. *J. Biochem. (Tokyo)* 99: 1699 (1986).
134. Sasaki T; Sugiyama N; Iwamoto M; Isoda S. *Chem. Pharm.Bull. (Tokyo)* 35: 2810 (1987).
135. Wiman B; Collen D. *Nature* 272: 549–50 (1978).
136. Aoki N; Sakata Y; Ichinose A. *Blood* 62: 1118–1122 (1983).
137. Tamaki T; Aoki H. Cross-linking of  $\alpha_2$ -plasmin inhibitor and fibronectin to fibrin by fibrin-stabilizing factor. *Biochim Biophys Acta* 661: 280–6 (1981).
138. Ichinose A; Aoki N. Reversible cross-linking of alpha 2-plasmin inhibitor to fibrinogen by fibrin-stabilizing factor. *Biochim Biophys Acta* 706: 158–64 (1982).

139. Sakata Y; Aoki N. Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J Clin Invest* 65: 290–7 (1980).
140. Ichinose A; Aoki N. Reversible cross-linking of alpha 2-plasmin inhibitor to fibrinogen by fibrin-stabilizing factor. *Biochim Biophys Acta* 706: 158–164 (1982).
141. Saito H. *J. Lab. Clin. Med.* 112: 671 (1988).
142. Favier R; Aoki N. de Moerloose, P. *Br. J. Haematol.* 114: 4 (2001).
143. Mosesson MW; Siebenlist KR; Hernandez I; Lee KN; Christiansen VJ; McKee PA. Evidence that  $\alpha_2$ -antiplasmin becomes covalently ligated to plasma fibrinogen in the circulation: a new role for plasma factor XIII in fibrinolysis regulation. *Thromb. And Haemos* 6: 1565-1570 (2008).
144. Zardi L; Cecconi C; Barbieri O; Carnemolla B; Picca M; Santi L. Concentration of fibronectin in plasma of tumor-bearing mice and synthesis by Ehrlich ascites tumor cells. *Cancer Res* 39: 3774-3779 (1979).
145. Mao Y; Schwarzbauer JE. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol* 24: 389-399 (2005).
146. Mosesson M. W.; Chen A. B.; Huseby R. M. *Biochim* (1975).
147. Mosher D. F. *J. Biol. Chem.* 260: 6614-6621 (1985).
148. Kurkinen M.; Vartio T.; Vaheri, A. *Biochim. Biophys. Acta* 624: 490-498 (1980).
149. Furie M. B.; Rifkin D. B. *J. Biol. Chem.* 255: 3134-3140 (1980).
150. Skorstengaard K.; Thegersen H. C.; Vibe-Pedersen K.; Petersen I. E.; Magnusson S. *Eur. J. Biochem.* 128: 605-623 (1982).
151. Odermatte; J. Engel. Physical properties of fibronectin. In Fibronectin. D. F. Mosher, Ed.: 25-43. *Academic Press*. New York, NY (1989).
152. Petersen T. E.; Thogerson H. C.; Skorstengaard K.; Vibe-Pedersen K.; Sahl P.; Sottrup-Jensen L.; Magnusson S. *Proc. Natl. Acad. Sci. U. S. A.* 80: 137–141 (1983).
153. Rocco M.; Carson N.; Hantgan R.; McDonagh J.; Hermans J. *J. Biol. Chem.* 258: 14545–14555 (1983).
154. Leahy D. J.; Aukhil I.; Erickson H. P. *Cell* 84: 155–164 (1996).
155. Deanef. Mosher; Ofrances J. Fogerty; Michael A.Chernousov; Elizabeth L; R. Barry. Assembly of Fibronectin into Extracellular Matrix. *Adhesive Proteins and the Extracellular Matrix*; Part V.
156. Hynes RO. Fibronectins *Springer-Verlag*, New York (1990).
157. Burns GF;C osgroveL; TrigliaT; BeallJ A; L6pezA F; Werk-meister JA; Begley CG; Haddad AP; d'Apice AJF; Vadas MA; CawleyJ C. The IIb-IIIag lycoprotein complex hat mediate sp lateleta ggregationis directlyim plicatedin leu-kocyte adhesion. *Cell* 45: 269-80 (1986).
158. Mosher DF; Johnson RB. In vitro formation of disulfide-bonded fibronectin multimers. *J Biol Chem* 258: 6595–6601 (1983).
159. Matsuka YV; Migliorini MM; Ingham KC. Cross-linking of fibronectin to C-terminal fragments of the fibrinogen  $\alpha$ -chain by factor XIIIa. *J Protein Chem* 16: 739–745 (1997).

160. Makogonenko E; Tsurupa G; Ingham K; Medved L. Interaction of fibrin(ogen) with fibronectin: further characterization and localization of the fibronectin-binding site. *Biochemistry* 41: 7907–7913 (2002).
161. Quade B. J.; McDonald J. A. *J. Biol. Chem.* 263: 19602-19609 (1988).
162. Patti J. M.; Allen B. L.; McGavin M. J.; Ho"o"ok, M. *Annu. ReV. Microbiol.* 48: 585-617 (1994).